Universidade do Minho
Escola de Engenharia

Ana Margarida Arantes Pereira

Development of a voltammetric aptasensor for the detection of proteins with biomedical relevance

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Trabalho realizado sob a orientação da
Doutora Lígia Rodrigues
e do
Mestre Sofia Meirinho

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TÍTULO DA DISSERTAÇÃO DE MESTRADO:

Development of a voltammetric aptasensor for the detection of proteins with biomedical relevance

Desenvolvimento de um aptasensor voltamétrico para a deteção de proteínas com relevância biomédica

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_____

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Abstract

Translating biomedical knowledge of biomarkers into clinically relevant devices that could be used as diagnostic or monitoring tools for disease management using effective analytical techniques is extremely important and still remains a challenge. However, the existing analytical methods for real-time protein detection in homogeneous solutions are limited.

In this research, molecular aptamers were combined with fluorescence techniques and electrochemical sensors to provide an easy and efficient method to detect proteins. In a first step, two types of high-affinity thrombin-binding aptamers (TBA1 and TBA2) and OPN-R3, an aptamer that has been reported to bind specifically to human osteopontin, were labeled with 6-FAM and used as molecular recognition probes to conduct ELISA experiments with human thrombin, human osteopontin and interferences such as bovine osteopontin and bovine serum albumin. Through non-linear fitting it was found a dissociation constant value of 1.820 nM and 0.867 nM for TBA1 and TBA2, respectively, and 5.65 nM for OPN-R3. This constant, K_d is commonly used to describe the affinity between a ligand and a protein i.e. how tightly a ligand binds to a particular protein. An Electrophoretic Mobility Shift Assay (EMSA) was also conducted to validate the formation of the aptamer:protein complexes, however the results obtained were inconclusive, possibly due to the low protein concentrations used.

The ultimate goal of this work was to develop a voltammetric aptasensor for the detection of thrombin, using the aptamer as the detection probe and $[Fe(CN)_6]^{4-/3-}$ as the electrochemical active redox solution. Appropriate aptamer sequences (TBA1 and TBA2) were designed to enable binding to thrombin, and also to include a biotin molecule in the 5'-end for the immobilization step. The aptamers were immobilized through the avidin-biotin methodology on screen-printed electrodes adequate for electrochemical detection. Thrombin detection was studied using cyclic voltammetry. The aptasensor presented a linear response for thrombin concentrations in the range between 0.5 nM and 50 nM, and a detection limit of 0.025 nM. Furthermore, this aptasensor was found to be specific for thrombin. The results gathered in this thesis are promising, suggesting that aptasensors constitute an alternative approach for the detection of proteins with biomedical relevance.

Resumo

O desenvolvimento de dispositivos que constituam ferramentas de diagnóstico e monitorização de doenças, fazendo recurso ao conhecimento existente sobre biomarcadores e usando técnicas analíticas eficazes, é actualmente uma área de grande interesse e relevância. No entanto, as técnicas analíticas disponíveis para a deteção de proteínas em tempo real e em soluções homogéneas são ainda limitadas.

Neste trabalho de investigação, os aptámeros moleculares foram combinados com técnicas de fluorescência e sensores eletroquímicos para desenvolver uma abordagem simples e eficiente para a deteção de proteínas. Numa primeira etapa, dois tipos de aptámeros de elevada afinidade de ligação à trombina (TBA1 e TBA2) e o aptámero OPN-R3, que foi reportado por se ligar especificamente a osteopontina humana, foram marcados com 6-FAM e usados como sondas de reconhecimento molecular para conduzir ensaios ELISA com trombina e osteopontina humana e interferentes como osteopontina bovina e albumina de soro bovina. Através do ajuste não linear dos dados foi possível determinar um valor para a constante de dissociação de 1.820 nM e 0.867 nM para TBA1 e TBA2, respectivamente, e 5.65 nM para o OPN-R3. Esta constante, K_d é usada para descrever a afinidade entre um aptámero e uma proteína, ou seja, saber quão forte é a ligação entre o aptámero e a respectiva proteína. Adicionalmente, foram realizados ensaios de mobilidade electroforética, a fim de validar a formação do complexo aptámero:proteína, no entanto os resultados obtidos foram inconclusivos, possivelmente devido às baixas concentrações de proteína usadas.

O principal objetivo deste trabalho foi desenvolver um aptasensor voltamétrico para a deteção de trombina, usando o aptámero como sonda de detecção e $[Fe(CN)_6]^{4-/3}$ como solução redox electroquímica. Foram desenhadas sequências de aptámeros marcados com uma molécula de biotina na sua extremidade 5' para auxiliar o passo de imobilização. Estas sequências: TBA1 e TBA2 ligam especificamente à trombina. Os aptámeros foram imobilizados usando a metodologia da avidina-biotina em eléctrodos desenhados especificamente para a deteção electroquímica. A detecção da trombina foi estudada por voltametria cíclica. O aptasensor apresenta uma resposta linear para concentrações de trombina na gama entre 0.5 nM e 50 nM, e um limite de detecção de 0.025 nM. Adicionalmente, verificou-se que o aptasensor é específico para a trombina. Os resultados desta tese são bastante promissores, sugerindo que os aptasensores podem constituir uma abordagem alternativa para a deteção de proteínas com relevância biomédica.

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Abbreviations

CA15-3	Biomarker analyzed in breast cancer patients
CEA	Carcinoembryonic antigen
CA-125	Elevated cancer antigen
RT-PCR	Reverse transcription – polymerase chain reaction
CA 19-9	Biomarker analyzed in pancreatic and gastrointestinal cancer patients
AFP	Biomarker analyzed in testis, ovary and liver cancer
MIF	Biomarker analyzed in mammary tumors
OPN	Osteopontin
Thr	Thrombin
PSA	Prostate-specific antigen
NCBI	National Centre for Biotechnology Information
BC	Breast Cancer
IUPAC	International Union of Pure and Applied Chemistry
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
IL-1	Interleukin-1
PCR	Polymerase chain reaction
SELEX	Systematic Evolution of Ligands by Exponential enrichment
FDA	Food and Drug Administration
AMD	Macular Degeneration Treatment
VEGF	Vascular Endothelial growth factor
TBA 1	Thrombin binding aptamer 1
TBA 2	Thrombin binding aptamer 2
R3-OPN	Osteopontin-binding RNA sequence
ELISA	Enzyme-Immunosorbent Assay
FA	Fluorescence Anisotropic
EMSA	Electrophoretic Mobility Shift Assays
ISFETS	Ion-sensitive field-effect transistors
SAM	Self- Assembled Monolayers
LBL	Layer- by- Layer
Fc- PEI	Ferrocene-appended poly(ethyleneimine)
CNT`s	Carbon Nanotubes
SPE	Screen- printed electrodes
Kd	Dissociation Constant
EDC / NHS	1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide sulfosuccinimide
QCM	Quartz crystal microbalance
SA	Sandwich Assay
EIS	Electrochemical impedance spectroscopy
TID	Target- Induced Displacement Mode
QD`s	Quantum Dots
NP`s	Nanoparticles
LOD	Limit of Detection
PBS	Phosphate Buffer Saline
MB	Methylene Blue
GDH	Glucose dehydrogenase
MG	Methylene green
Fc	Ferrocene
CDs	Cyclodextrins

RT	Room temperature
FAM	Fluorescein
F_T	Total fluorescence
F_{APTNB}	Fluorescence of the aptamer not bound
$F_{APTBOUND}$	Fluorescence of the aptamer bound
$F_{COMPLEX}$	Fluorescence of the complex
APS	Ammonium persulfate
TEMED	(N,N,N′,N′-Tetramethylethylenediamine
TBE	Tris-borate-EDTA
DSP	3,3`-dithiopropionic acid-di(N-succinimidylester
DMSO	Dimethyl sulfoxide
BSA	Bovine serum albumin
CV	Cyclic voltammetry
rhOPN	Human recombinant osteopontin
bOPN	Bovine serum osteopontin
hThr	Human thrombin

1. Introduction

Current demands regarding human health have encouraged the development of an increasing number of clinical tests. As a result, there is a growing requirement to develop more sensitive, reliable, time-efficient and inexpensive methods of analysis (Hong, Li, & Li, 2012). Despite the outstanding progresses in the field of biomedicine, substantial challenges remain in translating biomedical knowledge on disease markers into clinically relevant devices that could be used as diagnostic or monitoring tools for disease management (X. Y. Wang, Gao, Lu, He, & Yin, 2013). Progress in the development of consistent malignancy markers is imminent due to the advances in genomics and bioinformatics fields that enabled the analysis of great amounts of data through the use of less complex procedures involving less wet-lab analysis, and also with a lower requirement for qualified and experienced technical staff to conduct the analysis (Opstal-van Winden et al., 2011; Rodrigues, Teixeira, Schmitt, Paulsson, & Lindmark-Mänsson, 2007). The adoption of high-throughput methods led to the discovery of many new biomarkers that have been reported for prognostic and predictive purposes. However, out of these only a few have made their way into clinical routine mainly due to the lack of sufficient validation (Bohunicky & Mousa, 2010).

A biomarker is an indicator of a biological state or condition which, besides its value for diagnosis purposes, can be helpful to evaluate the body reaction to a given treatment (Bohunicky & Mousa, 2010; J. Wang, Chen, Jiang, Li, & Wang, 2013). The use of biomarkers to anticipate the outlines of the disease has been an emerging issue, especially in the case of cancer for which several treatments have been quite successful in the last few years. Biomarkers can also provide information on the mechanism underlying the initiation of a disease, and ultimately they constitute a powerful tool to precisely define the disease states, as well as to define appropriate treatments even for early stages of diseases (J. Wang et al., 2013). Some of the biomarkers that undergone rigorous testing are summarized in Table 1.

Table 1 List of biomarkers tested. Adapted from: Polanski & Anderson, 2006; Tothill, 2009; Opstal-van Winden et al., 2011 and Bohunicky & Mousa, 2010

Biomarker	Application
CA15-3	Monitoring marker breast cancer
CEA	Monitoring marker breast cancer
CA-125	Monitoring marker breast cancer and ovarian cancer
CA19-9	Monitoring marker pancreatic and gastrointestinal cancer Increased serum levels in human breast cancer cases
AFP	Staging marker testis, ovary and liver cancer Increased serum levels in human breast cancer cases
MIF	Higher expression in mammary tumors compared to normal tissue Increased serum levels in human breast cancer cases
Prolactin	Risk marker for breast cancer
Leptin	Higher expression in mammary tumors compared to normal tissue Risk marker for breast cancer Differential plasma levels in human breast cancer cases
OPN	Higher in humanized Mouse models for breast cancer Increased plasma and serum levels in plasma breast cancer cases Increasing expression in mammary tumor tissue and plasma in progressing disease
Haptoglobin	Higher in humanized Mouse models for breast cancer Increased serum levels in human breast cancer cases
PSA	Screening and Monitoring marker prostate cancer
B-type natriuretic peptide	Marker of congestive heart failure
Bladder Tumor Antigen	Marker for urothelial carcinoma

The discovery of new biomarkers is a time-consuming and costly task, since it requires the systematic separation and identification of biological molecules from complex body fluids (e.g. urine, blood) or tissues (Bohunicky & Mousa, 2010; Medley, Bamrungsap, Tan, & Smith, 2011;

Phillips, Xu, Xia, Fan, & Tan, 2009). Biomarkers that have been recognized as reliable for disease diagnosis and prognosis are listed in several reference cancer databases (e.g. National Centre for Biotechnology Information (NCBI), Cancer Research United Kingdom) and these have been approved by the FDA (Food and Drug Administration) (Opstal-van Winden et al., 2011; Tothill, 2009). Moreover, guidelines have also been developed describing the best approach to report the discovery of novel tumor markers, in order to guarantee that such studies are clear and easily understood in the context of their conclusions (McShane et al., 2005).

Biomarkers can either be present inside the cancer cells or be extracellular. Besides, they can also be of various molecular origins, including DNA (i.e. specific mutation, translocation, amplification, and loss of heterozygosity), RNA, or protein (i.e. hormone, antibody, oncogene or tumor suppressor)(Bohunicky & Mousa, 2010). Proteins that are selectively overexpressed as the result of cancer cells growth have been selected as potential biomarkers for cancer diagnosis and/or prognosis. Thus, these proteins can provide the basis for screening techniques, treatment options (Medley et al., 2011; Tothill, 2009), metastasis evaluation, and for determining the response to pharmacologic intervention (Sadana & Sadana, 2011).

Several proteins in the blood were indeed found to be related with the presence of breast cancer (J. Li et al., 2005; Lord et al., 2007; Mathelin, Cromer, Wendling, Tomasetto, & Rio, 2006). Based on the hypothesis that these proteins can be useful biomarkers for the early detection of cancer, great efforts have been developed to discover such proteins that are overexpressed in patient's body fluids (Misek & Kim, 2011).

During the past decade, emerging evidence has refined the value of osteopontin (OPN) as a potential biomarker. OPN is an extracellular matrix protein with adhesive properties, that possesses a thrombin cleavage site and a cell attachment sequence found in all body fluids as plasma and serum (D. X. Cao et al., 2012; Fisher, Torchia, Fohr, Young, & Fedarko, 2001; Higashikawa, Eboshida, & Yokosaki, 2007; Ke et al., 2011; Macrì et al., 2009). Current research showed that OPN is often overexpressed in human cancers and contributes to regulate tumor growth and progression (Rangaswami, Bulbule, & Kundu, 2006; Rittling & Chambers, 2004; Servais et al., 2011). Increased OPN expression is one of the characteristics often associated with metastatic cancer cells (Kadkol et al., 2006; Rudland et al., 2002; P. Y Wai & Kuo, 2004), and it has been suggested as a main player in the progression and metastasis of a variety of cancers, including breast, liver, prostate and lung (Shojaei et al., 2012; P. Y. Wai & Kuo, 2008; Weber, Lett, & Haubein, 2010). Beausoleil and co-workers (2011) demonstrated that the changes in the OPN

plasma levels after therapy and over time could be used to monitor the clinical outcome (Beausoleil, Schulze, Goodale, Postenka, & Allan, 2011).

Several recent studies describe the multiple and complex mechanisms in which OPN is involved and suggest it's potential as a biomarker for ovarian (J.-h. Kim et al., 2002; Matsuura, Suzuki, & Saito, 2010), bladder urothelial carcinoma (Park et al., 2012), gastric and liver (D. X. Cao et al., 2012), and for breast cancer (BC) (Mirza et al., 2008).

As mentioned, OPN contains two integrin-binding sites and a thrombin cleavage domain located in close proximity to each other. When OPN is cleaved at these domains by thrombin, it is separated into two approximately equivalent sized pieces, and since thrombin is also often overexpressed on the surface of cancer cells, the tumor microenvironment favors the activation of thrombin, and therefore the OPN cleavage (Beausoleil et al., 2011).

Human thrombin, highly specific serine protease, is also a biomarker that plays an important role on procoagulant and anticoagulant functions. This protein plays multiple roles in endothelial and smooth muscle cell functions, as well as coagulation and hemostasis (P. Wang et al., 2011). It is activated by the proteolytic cleavage of its precursor molecule pro-thrombin generation factor *Xa* (W. Z. Xie et al., 2005). Thrombin converts fibrinogen to insoluble fibrin that forms the fibrin gel, which is responsible either for some physiological plugs or for the formation of pathological thrombus (Neundlinger et al., 2011). The concentration of thrombin in blood varies considerably and it can be almost absent in the blood of healthy individuals. Some studies refer that its concentration in blood is normally around 0.01 nM (Frense et al., 2013). However, thrombin can reach low-micromolar concentrations during the coagulation process, and low levels of thrombin can even be generated in the early hemostatic process (Sosic, Meneghello, Cretaio, & Gatto, 2011).

In addition to its central role in coagulation, thrombin has been reported to induce mitogenesis and differentiation in cancer cells (W. Z. Xie et al., 2005); to regulate microvascular permeability (Guttridge, 1997); and also to be involved in blood coagulation, incrustation, inflammation and pulmonary metastasis (Chiu & Huang, 2009; Lin et al., 2011). Due to its crucial involvement in both thrombosis and hemostasis, thrombin is a major target for anticoagulation and cardiovascular disease therapy (Paborsky, N. McCurd, C. Griffin, J. Toole, & L. K. Leung, 1993).

Thrombotic disorders and their common clinical phenotypes of ischemic stroke, and venous thromboembolism cause substantial health care expenditures, morbidity, and mortality worldwide. The *in vivo* detection of thrombin, for example using aptamers, could be a promising method to prevent and/or treat these diseases (P. Wang et al., 2011).

1.1. Biosensors

Currently, there is a high demand for convenient methodologies that allow detecting and measuring the levels of specific proteins in biological and environmental samples. In general, their detection, identification and quantification using conventional techniques, such as molecular assays and microbial culture-based tests, can be very complex, expensive and time consuming (Hong et al., 2012).

A biosensor, *as per* definition of IUPAC, is an integrated receptor-transducer device, which is capable of providing bio-recognition processes into measurable signals via a physicochemical transducer, with electronic and optical techniques as two major transducers (Shiping Song, Xu, & Fan, 2006; Beate Strehlitz, Nikolaus, & Stoltenburg, 2008). The use of biosensors brings about a combination of advantages. First, biosensors are highly sensitive, mainly because biomolecules often possess high affinity towards their targets. Second, biological recognition is usually very selective, thus it often leads to selective biosensors. Third, arising due to the development of modern electronic industry, it has been relatively easy to develop inexpensive, integrated and ready-to-use biosensor devices. These biological sensors certainly improve the ability to detect molecules or pathogens, besides being of particularly usefulness for small clinics and even for point-of care analysis (K. M. Song, Lee, & Ban, 2012; Shiping Song et al., 2006).

The core design of a biosensor mainly includes three components namely the probe-target recognition (that detects the presence or concentration of biological molecules or biological structures), the signal transduction (which translates the biochemical interaction into a quantifiable physical signal) and the physical readout (Bohunicky & Mousa, 2010; D'Orazio, 2003; J. O. Lee et al., 2008), as illustrated in Figure 1.

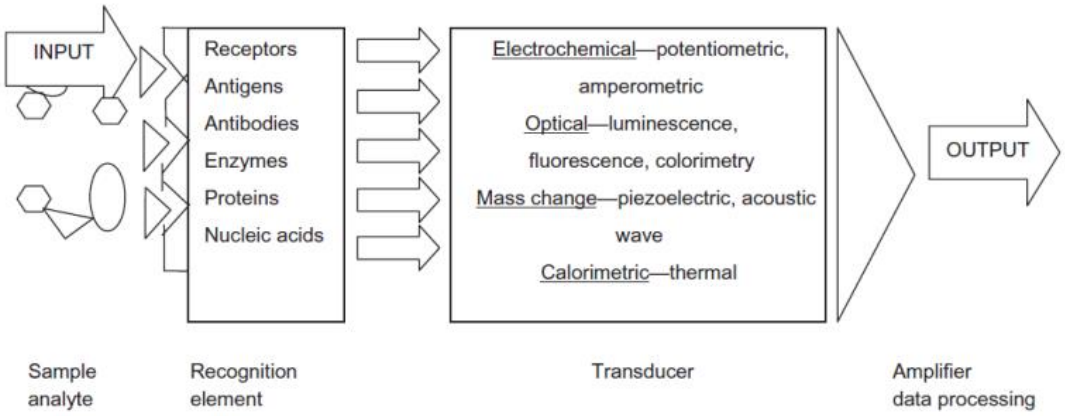

Figure 1 Illustration of a biosensor functioning process. Taken from Bohunicky & Mousa, 2010

1.1.1. Biorecognition element - Aptamers

The biorecognition element is a critical key component of any biosensor. Fortunately, due to advances in technology and synthetic chemistry, many biosensor recognition elements used today are synthesized in the laboratory for improved stability and reproducibility of the biosensor function. Examples of recognition elements include receptor proteins, antigens, antibodies, enzymes, and nucleic acids (specifically aptamers) (Bohunicky & Mousa, 2010).

Aptamers (from the Latin word "aptus" meaning to fit and the Greek word "meros" meaning particle or piece) (Mairal et al., 2008; Mayer, 2009) are artificial nucleic acid ligands which can be selected from combinatorial libraries of synthetic nucleic acids, possessing motifs that recognize their targets (Hong et al., 2012). These molecules are 20–80 base pair long single-stranded ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) oligonucleotides which are folded into distinct three-dimensional conformations (Kanwar, Roy, & Kanwar, 2011). Binding occurs because of their specific and complex three-dimensional shape characterized by loops, hairpins, stems, bulges, pseudoknots, triplexes, or quadruplexes. The structure compatibility results in the aptamer-target binding by stacking of aromatic rings, van der Waals and electrostatic interactions, and hydrogen bonding, or from a mixture of these effects (B. Strehlitz, Reinemann, Linkorn, & Stoltenburg, 2011).

Aptamers are similar to antibodies concerning their binding affinities; however they possess unique features, which turn them into potential alternatives to the antibodies. Firstly, the aptamers are generated using *in vitro* settings contrarily to the antibodies that are generated *in vivo* (Jayasena, 1999; J. H. Lee, Yigit, Mazumdar, & Lu, 2010). *In vitro* selection allows oligomers to be screened against molecules that have weak immunogenicity or high toxicity (Jayasena, 1999; J. H. Lee et al., 2010; Tombelli, Minunni, Luzi, & Mascini, 2005). In addition, non-physiological and/or harsh conditions can be applied while selecting aptamers (Jayasena, 1999; J. H. Lee et al., 2010; Tombelli et al., 2005). For instance, the thermal denaturation of nucleic acids is reversible, as exhibited in polymerase chain reaction (PCR), whereas antibodies or proteins such as interleukin-1 receptor (IL-1) are permanently denatured at temperatures higher than 53.5 °C (Remmele et al., 2005). Secondly, since the method for the synthesis and purification of oligonucleotides is well developed, the batch-to-batch difference of aptamer products is minimal compared with that of antibodies (Jayasena, 1999; J. H. Lee et al., 2010; Tombelli et al., 2005). Also, the aptamers can be chemically modified with a variety of functional groups and/or fluorophores without loss of binding functionality (J. H. Lee et al., 2007). Besides, as mentioned above, aptamers are structurally versatile because they have basic stem-loop arrangements that form proper three-

dimensional structures. These structures facilitate the formation of a complex with the target molecule. Thus, aptamers have high affinities to their targets, with dissociation constants at the low picomolar level, comparable to or better than antibodies (Gopinath, Awazu, & Fujimaki, 2012).

Furthermore, aptamer-based ligands may exhibit prominent advantages that include site-specific labeling, structure-controlled design and sequence-dependent amplification, which makes them an ideal molecular recognition tool for the development of biosensors (Zhang, Huang, Jiang, Shen, & Yu, 2007). In this context, besides the proliferated uses of aptamers instead of antibodies in established immunoassay techniques, the development of aptamer sensors with unique response strategies for homogeneous assays has been a subject of intensive research (Cho, Lee, & Ellington, 2009).

In addition, aptamers can be easily labeled for their use in diagnostics (Ulrich & Wrenger, 2009). As the use of aptamers has been extended from basic biology of cellular processes and gene regulation to therapeutic and diagnostic applications, many patented aptamers are currently being tested in clinical trials and have been recently reviewed (Kanwar et al., 2011). One of the major challenges in this field is the development of general methods to convert the highly specific molecular recognition between aptamers and their targets into detectable signals. Conjugation of aptamers with labels, nanoparticles, enzymes, among others, can be an ideal way to overcome this hurdle (Y. S. Kim, Lee, & Gu, 2008).

Systematic Evolution of Ligands by Exponential enrichment (SELEX) methodology is a combinatorial chemical procedure, commonly known as "*in vitro* selection" that allows the identification of nucleic acid sequences with unique properties from a random pool of sequences (an aptamer library), which were described above as aptamers (Figure 2).

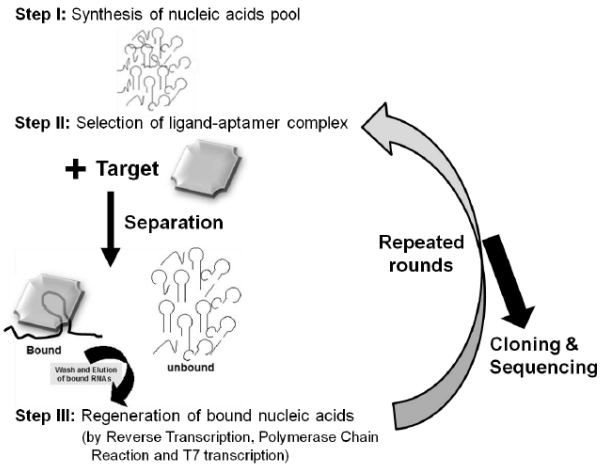

Figure 2 Isolation of aptamers using the SELEX procedure. Taken from Gopinath & Fujimaki, 2012

Since then, the SELEX methodology has been used to identify aptamers with high-affinity and specificity against hundreds of targets, becoming an important research tool especially in the discovery of new drugs and disease targets, clinical diagnosis, therapy and in food inspection. There have been attempts to search for aptamers that are specific to targets involved in various diseases, such as cancer and viral infection (K. M. Song et al., 2012). Developed aptamers have been studied primarily for applications as diagnostic or therapeutic tools. In 2004, the Food and Drug Administration (FDA) approved the first RNA aptamer (*Macugen, Pegaptanib*) for human use in age-related macular degeneration treatment (AMD) targeting the vascular endothelial growth factor (VEGF) (Bunka & Stockley, 2006; Djordjevic, 2007; K.-T. Guo, Paul, Schichor, Ziemer, & Wendel, 2008). SELEX was used in many of these cases to identify dsDNA sequences that are the strongest (consensus) binders to the protein of interest (Djordjevic, 2007).

The choice between DNA or RNA aptamers depends essentially on practical considerations, and in part on the final application envisaged. In terms of a wide conformational diversity, RNA aptamers are naturally more flexible than the DNA molecules, but the RNA is more susceptible to degradation by nucleases which restricts their use in the presence of biological fluids. In terms of stability, using chemical modifications of the nucleotides, DNA aptamers are considered naturally more robust and display high levels of stability for biological applications than RNA aptamers. (Kulbachinskiy, 2007). Several strategies have been proposed to overcome the issue of RNA aptamers degradation, including their modification with 2′-aminopyrimidine, 2′-fluoropyrimidine or 2′-O-methyl nucleotides to increase their stability (Burmeister et al., 2005; Chiu & Huang, 2009; Feng et al., 2008; Keefe & Cload, 2008; Mayer, 2009; Stoltenburg, Reinemann, & Strehlitz, 2007).

Virtually, aptamers can be raised against any target. This feature makes them interesting molecules to recognize for example proteins that have been previously identified as biomarkers. One of the most well-known examples of *in vitro* selection of DNA oligonucleotides for targeting a specific protein is the thrombin-binding aptamer (TBA). This thrombin aptamer has been extensively studied and characterized, thus enabling the monitoring of thrombin levels in plasma or blood (Diculescu, Chiorcea-Paquim, Eritja, & Oliveira-Brett, 2010) It has been shown that in the presence of alkali metals, TBA forms an antiparallel quadruplex consisting of two G-quartets connected by two TT loops and one TGT loop. (Bock, 1992). In particular, the thrombin binding aptamer 1 (TBA1) and 2 (TBA2) consist of two G-quartet conformations that selectively bind to specific and different epitopes of human α-thrombin (Tasset, Kubik, & Steiner, 1997) (Figure 3). TBA1 is a 15-mer DNA aptamer which binds exosite I of thrombin (Fibrinogen Binding Site), with a dissociation constant (Kd) of approximately 26 nM (Bock, 1992; Paborsky et al., 1993; Tasset et al., 1997). TBA2 is a 29-mer

DNA aptamer binding to exosite II of thrombin (Heparin Binding Domain) with subnanomolar affinity (Sosic et al., 2011). This distinct recognition pattern allows their use in tandem, since a ternary complex could possibly be formed by simultaneous recognition of thrombin with different formats and detection methods have been demonstrated by few groups (Bang, Cho, & Kim, 2005; D. W. Huang, Niu, Qin, Ruan, & Zeng, 2010; A.-E. Radi, Sánchez, Baldrich, & O`Sullivan, 2005; Zhao et al., 2011)

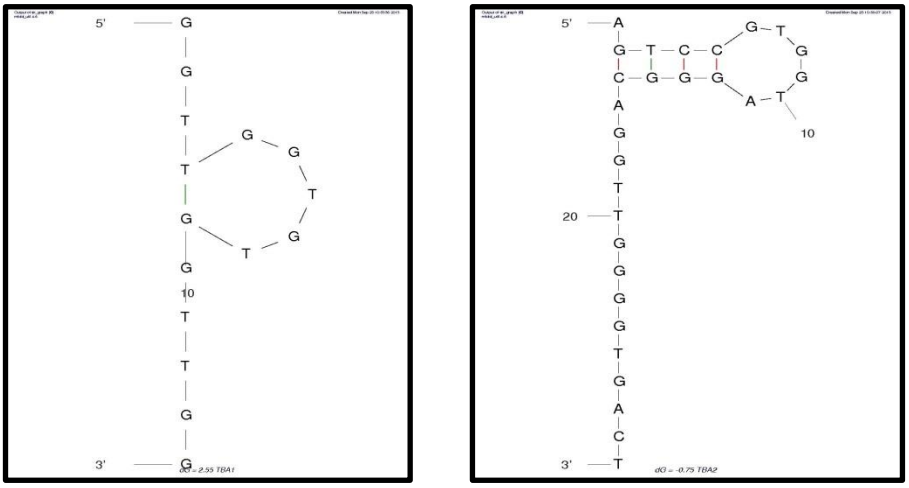

Figure 3 Schematic representation of the G-quartet of (a) TBA1 and (b) TBA2 (by Mfold web server)

Some studies have reported the use of TBA to detect thrombin as summarized in Table 2. In most cases, it appears that the methodology adopted has been strongly influenced by the authors' background, usually taking advantage of ELISA or nucleic acid-related protocols, as described below in Table 2. Discrepancies in values may also arise from the fact that each aptamer has a unique structure for target binding (E. Baldrich, Restrepo, & O'Sullivan, 2004).

Table 2 Assays reported from different authors in which TBA has been used. Adapted from Baldrich, E. et al., 2004 and Centi, S. et al., 2007

TBA Labelling and immobilization methods	Assay Format	Detection	Limit of detection (LOD)	Dissociation Constant (K_d)	Reference
In solution 15-mer and 29-mer	Magnetic Beads coated with StreptAvidin	Electrochemical	0.45 nM	15-mer: 26nM and 29-mer: 0.5 nM	(Centi, Tombelli, Minunni, & Mascini, 2007)
In solution	Direct polymeric staining	Change in color due to TBA folding	10pM		(Ho & Leclerc, 2004)
In solution (5`-fluorphore 3`-quencher)(2 fluorophore)	Molecular beacon	Change in fluorescence	373 pM 429pM	5.2±0.49nM	(J. J. Li, Fang, & Tan, 2002)
In solution (5`-fluorphore 3`-quencher)	Molecular beacon	Change in fluorescence		10nM	(Hamaguchi, Ellington, & Stanton, 2001)
5`-NH-C6 on silica beads optic fibers (microarray)	Competitive reusable	Change in fluorescence (FITC-thrombin)	1nM	300nM	(M. Lee & Walt, 2000)
3`-C7 glass slide immobilized, 5`-FITC	Molecular beacon reusable	Evanescent wave-induced fluorescence anisotropy	5nM	47nM	(Potyrailo RA, Conrad RC, Ellington AD, & GM., 1998)

An OPN-directed RNA aptamer, OPN-R3, was recently isolated through SELEX technology. Binding studies with human OPN, were conducted using the OPN-R3 aptamer (K_d value of 18 ± 0,2 nmol/l) and an unspecific competitor RNA aptamer. It was found that human OPN binds to OPN-R3 in a specific manner (Zhiyong Mi et al., 2009). Besides, further research indicated that RNA aptamer binding to OPN blocks its interaction with cancer cell surface receptors to significantly

inhibit adhesion, migration and invasion *in vitro,* thus inhibiting local progression and distant metastases (Mi, Guo, & Kuo, 2009).

1.1.2. Transducer

Regarding the physical transducer, it is the part of the device that converts the recognition signal events into electrical (often digital) signals – biosensors can be categorized in various types, including resonant, photometric, thermal detection, ion-sensitive field-effect transistors (ISFETs), and electrochemical sensors (Tothill, 2009; Y. Zhou, Chiu, & Liang, 2012). Table 3 summarizes the sensing mechanism of each type of biosensor.

Table 3 Types and sensing mechanisms involved in different biosensor. Adapted from: Tothill, 2009 and Zhou, Y. et al., 2012

Type	Sensing mechanism	Transducer	Measured property
Resonant	The change of the viscosity, mass leads to change of resonant frequency of the acoustic wave	Mass sensitive	Resonant frequency
Thermal detection	Bio-reaction results in exothermic character	Thermal	Heat of reaction or adsorption
Photomeric	The change of refractive of the solution leads to the change of refractive angle of the incident light	Optical	Surface Plasmon resonance angle, Fluorescence
ISFETs	The ionic analyte diffuses into the membrane hence change the potential difference at the detecting interface	Ion-selective membrane	Surface potential
Electrochemical	Bio-reaction results in ions production or consumption will create the charge transfer across the double layer of the transducer	Electrochemical	Potentiometric Amperometric Impedimetric

1.1.2.1. Techniques for modification of surfaces: Self-assembled monolayers (SAM) and Layer-by layer (LBL)

Self-assembled monolayers (SAM) and Layer-by-Layer (LBL) are methodologies that lead to the deposition of organic compounds on the biosensor surfaces. Among the commonly used substrates, gold presents some benefits for many electrochemical measurements. The gold surface can be functionalized and the type of chemistry selected is dependent on the type of terminal functional group linked to the aptamer (amine, thiol or biotin termini) (Velasco-Garcia & Missailidis, 2009). In addition, gold surfaces can be functionalized with SAM or LBL.

SAM provides molecular level control over the density and position of assembled molecules. SAM is capable of packing different types of molecules in an orderly fashion at the molecular level, which generates a multifunctional surface for multitasks. This layer of biological/chemical molecules is advantageous due to its simplicity of preparation, high sensitivity, and few limitations in the detection range of an analyte, and most importantly, the versatility of the modification that no other organic materials could match (Y. Zhou et al., 2012). As a substrate for monolayers assembly, gold presents interesting features: it is air-stable and commercially available from several sources as films and particles; it binds thiols with a high affinity (Balamurugan, Obubuafo, Soper, & Spivak, 2008), and the films are stable in complex liquid media containing target biomolecules. The monolayer can be designed to prevent non-specific adsorption of aptamers to the gold surface and it plays an important role in applications that require long-term storage stability (Kuralay, Campuzano, & Wang, 2012).

On the other hand, the layer-by-layer (LBL) approach is a convenient technique for the bottom-up assembly of multi-layered polymer films, because it allows the deposition of oppositely charged polyelectrolytes onto solid substrates (Du et al., 2010). The nature of the assembly process leads to precise, nanoscale control of film thickness and composition through the appropriate choice of the components, the number of layers, and the order of their deposition (Sultan, Walsh, Monreal, & DeRosa, 2009; J. Wang, 2006; S. Xie & Walton, 2010). This method is highly versatile since several polyelectrolytes can be chosen. Biological macromolecules such as polypeptides/proteins, polysaccharides, nucleic acids and even viruses can serve as building blocks for these multilayer films. Sultan *et al.* (2009) reported that when embedded in or attached to LBL polyelectrolyte matrix, aptamers could maintain their affinity and specificity for the cognate target. The LBL technique has been reported for protein detection using self-assembled multilayers with ferrocene-appended poly(ethyleneimine) (Fc-PEI), carbon nanotubes (CNTs), and aptamers (Du et al., 2010).

Compared with SAM, these LBL multi-layers with three dimensional structure can bring in, not only more probes to produce an amplified signal, but also more molecular recognition elements to improve the sensitivity of the detection, making the stable membrane property of the multi-layer widely used in biosensors (Du et al., 2010).

1.2. Electrochemical detection

Electrochemical transducers are the most widely used in platforms for patient diagnosis since they are portable, simple, easy to use, low cost, minimal simple-to-operate, robust power requirements and independence of sample turbidity and in most cases disposable (A.-E. Radi, 2011; F. Wei, Lillehoj, & Ho, 2010). The electrochemical instruments used with the biosensors have been miniaturized to small pocket size devices which make them applicable for "alternative-site" testing, emergency-room screening, bedside monitoring or home self-testing (Tothill, 2009; J. Wang, 2006).

The name electrochemical biosensor is applied to a molecular sensing device which intimately couples a biological recognition element to an electrode transducer, in which the purpose of the transducer is to convert the phenomenon into a suitable and readable electrical signal.

Potentiometric and amperometric biosensors are the two most common types of electrochemical biosensors. Potentiometric biosensors use ion-selective electrodes to detect an electrical response in the molecular recognition element (Han, Liang, & Zhou, 2010). A true potentiometric aptasensor has been recently reported. This device was based on poly(phenothiazine) conducting polymers electropolymerized on a glassy carbon electrode. Avidin-modified polymer surfaces obtained by direct electrostatic precipitation have then been used to immobilize biotinylated anti-thrombin DNA aptamers. Measurement of the difference in the potential of the sensor enabled the potentiometric thrombin detection in a concentration ranging from 10^{-9} to 10^{-6} M (Evtugyn, Porfireva, Hianik, Cheburova, & Budnikov, 2008).

Amperometric transducers measure the current that is produced when a potential is generated between two electrodes. Oxidation or reduction reactions produce a current, which can then be measured (J. Wang, 2006). Amperometric-based biosensors for cancer detection, using sequence-specific DNA as the recognition element, have an enormous potential in the diagnosis field (Ikebukuro, Kiyohara, & Sode, 2005). Other examples include the work by Ikebukuro and co-workers (2004) that developed the first electrochemical aptasensor with an amperometric sandwich-based biosensor based on glucose dehydrogenase-labeled signaling aptamers. The GlucoWatch™ is a glucose sensor that was developed to enable non-invasive, continuous measurement of blood glucose levels in diabetic patients. This device works like a wristwatch and takes blood glucose readings through the skin via reverse ion-transfer, a process whereby a small electrical signal brings glucose to the skin surface so that it can be measured.

Electrochemical aptasensors have been recently developed for multiplexed protein measurements. In fact, since most cancer diseases are associated with the presence of more than one tumor marker, developing an effective aptasensor for simultaneous measurement of co-existing tumor markers may be valuable to improve the detection accuracy and to deliver more precise information on diagnosis, prognosis and treatment (Zhao(a) et al., 2012).

Electrochemical sensors are part of an electrochemical cell that often consists of three electrodes. A distinctive three electrode electrochemical cell consists of a working (or indicator) electrode of a chemically stable solid, conductive material, such as gold; a reference electrode and an auxiliary electrode (Ronkainen, Halsall, & Heineman, 2010) as shown in Figure 4. The working electrode serves as the transduction element in the biochemical reaction, while the counter electrode establishes the connection with the electrolytic solution so that a current can be applied to the working electrode (Grieshaber, MacKenzie, Voros, & Reimhult, 2008; Beate Strehlitz et al., 2008). Electroactive reporters such as methylene blue (MB), ferrocene, ferrocene-bearing polymers, ruthenium complexes and $[Fe(CN)_6]^{4-/3-}$ are commonly used for signal transduction in electrochemical aptamer-based sensors. These reporters can be covalently conjugated to the aptamer itself, conjugated to a complementary oligonucleotide or indirectly linked to aptamers (Cho et al., 2009). Screen- printed electrodes (SPEs) have been used in several electrochemical DNA sensors due to their straightforward fabrication, high uniformity and material versatility (F. Wei et al., 2010). Therefore, SPEs can offer an inexpensive solution with the additional advantage of flexibility in the biosensor shapes (D. Wei, Bailey, Andrew, & Ryhanen, 2009). Screen-printed technology has been reported as an attractive method for mass production of sensors (Alonso-Lomillo, Dominguez-Renedo, Matos, & Arcos-Martinez, 2009; Tothill, 2009).

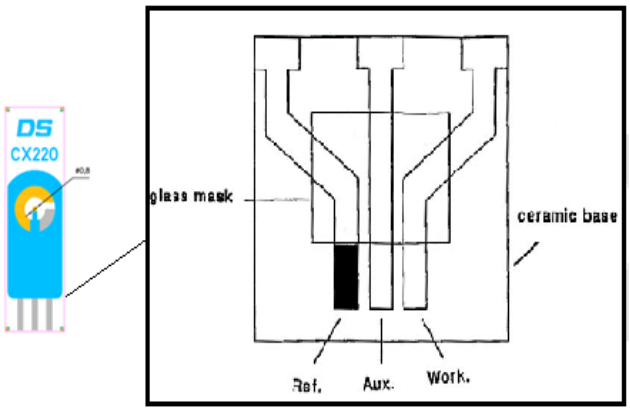

Figure 4 Screen-printed electrode with its diagram. Ref., reference electrode; Aux., auxiliary electrode; and Work, working electrode. Adapted from Ronkainen et al., 2008

1.2.1. Voltammetry

Voltammetry provides an electroanalytical method for gathering information on one or more analytes by measuring the current as a function of the potential in electrochemical aptasensors. Several types of experiments may be performed to collect information from voltammetry including cyclic voltammetry, square-wave voltammetry, and stripping voltammetry to name a few common techniques (Feng et al., 2008; Ikebukuro et al., 2005; K. M. Song et al., 2012; Beate Strehlitz et al., 2008; F. Wei et al., 2010; D. Xu et al., 2005).

Since there are many ways to vary a potential, there will also exist many forms of voltammetry, including polarography (DC Voltage), linear sweep, differential staircase, normal pulse, reverse pulse, differential pulse, among others. However, cyclic voltammetry is one of the most generally used forms and it is useful to obtain information about the redox potential and electrochemical reaction rates (e.g. the chemical rate constant) of analyte solutions.

The voltage is measured between the reference and the working electrodes, while the current is measured between the working and the counter electrode. The measurements are plotted as current *versus* voltage, also known as a voltammogram. The shape of the voltammogram for a specific target depends not only on the scan rate and the electrode surface, which is different after each adsorption step, but can also depend on the catalyst concentration (Grieshaber et al., 2008). Moreover, the detection can be either "signal on" or "signal off", depending on how the redox reporter is shielded from the electrode, resulting in maximum or minimum peaks, respectively on the voltammograms (Cho et al., 2009; Shiping Song, Wang, Li, Fan, & Zhao, 2008).

Additionally, through voltammetry it is possible to infer about some important parameters such as the biosensor affinity and probe-target binding; selectivity (especially important in real-world samples where the target concentration can be much less than the concentration of non-target biomolecules present); limits of detection and reproducibility (the detection limit can be determined by measuring the sensor response to a dilution series and determining the target smallest concentration at which the sensor response is clearly distinguishable from the response to a blank solution.); dynamic range (ratio between the largest measurable target concentration and the limit of detection); amplification (amplification techniques lay outside the domain of label-free impedance biosensors); and multiplexing (it means detecting several targets in the same biological sample is possible if different surface regions are functionalized with different probes) (Daniels & Pourmanda, 2007).

1.3. Immobilization methods

The critical step in the development of electrochemical aptasensors is the immobilization of the aptamers (biological recognition element) to the electrode surface. It is crucial to develop strategies for the reliable immobilization of aptamers so that they retain their biophysical characteristics and binding abilities, as well as for minimizing non-specific binding/adsorption events. In theory, these strategies are similar to those applied hitherto for the immobilization of single- or double-stranded DNA in DNA biosensors for the detection of DNA damage. As mentioned, the control of this step is essential to ensure high reactivity, orientation, accessibility, and stability of the surface-confined probe and to avoid non-specific binding (A.-E. Radi, 2011; Audrey Sassolas, Leca-Bouvier, & Blum, 2008).

Centi *et al.* (2007) stated that "The procedure to fix the aptamer to the surface is of paramount importance to obtain an ordered and oriented layer able to ensure, as much as possible, the flexibility of the bioreceptor without altering the affinity for the target molecule and selectivity that the aptamers show in solution"(Balamurugan et al., 2008). If immobilization is declined, it could lead to partial or complete loss of the target activity, due to possible structure deformation. Thus, to avoid this issue, proteins should be attached onto surfaces without affecting conformation and function (Rusmini, Zhong, & Feijen, 2007).

When developing an aptamer diagnostic device there are several different approaches for the immobilization of aptamers, which depend upon the chemical composition of the surface, the availability of suitable aptamer linkers, and the chemistries used for attachment (Balamurugan et al., 2008). Several substrates can be used as supports for the aptamer immobilization such as gold, silicates, carbon nanotubes (Fabre, Samorì, & Bianco, 2012; K. Guo et al., 2011) and polymers (L. Zhou, Wang, Wang, & Ye, 2011).

When developing an aptasensor, it is of utmost importance to define adequate immobilization strategies in order to maximize the performance of the aptamer-based analytical device (Balamurugan et al., 2008). Thus, in order to immobilize the aptamer onto the solid support it is required that either the 5′ or 3′ -end of the aptamer is modified (Gopinath et al., 2012). Although both positions can be used for the aptasensor development, there are very few studies looking at the effect of the two types of end attachment. Recent work suggests that it depends on the particular aptamer (Cho, Collett, Szafranska, & Ellington, 2006), although for biological targeting it may be that the 3' end is more suitable, since the 3' end is the primary target for exonucleases,

and thus its coupling to the solid support would simultaneously confer resistance to nucleases attack (Velasco-Garcia & Missailidis, 2009).

Few immobilization techniques for fixing aptamers onto biosensor surfaces have been developed in the past years, which are mainly based on the following three mechanisms: physical (adsorption), covalent (chemisorption with thiol and covalent attachment to chemically-modified surfaces by chemical groups such as hydroxyl, carboxyl and amino), and bio-affinity immobilization (using for example the Avidin-biotin interaction method) (Rusmini et al., 2007; Audrey Sassolas et al., 2008; L. Zhou et al., 2011) .

The choice of the best immobilization strategy is still an open question, although it is unlikely that one structure will be optimal for all proteins. Consequently, the extrapolation of immobilization strategies from one system to another for different classes of proteins is difficult and mostly unsuccessful due to the wide subset of characteristics and functional properties of proteins. Therefore, several unsolved challenges are involved in the immobilization of the aptamers (Rusmini et al., 2007).

1.4. Immobilization techniques

1.4.1. Physical adsorption

Physical adsorption is an immobilization method that does not requires any nucleic acid modification. It consists in placing the aptamer solution in contact with the surface for a well-defined period of time, and subsequently washing off any non-adsorbed biomolecules. It is based on the ionic, hydrophobic and Van der Waal's forces that take part in the interaction and will depend on the particular protein and surface involved. The resulting layer is likely to be heterogeneous and randomly oriented, since each molecule can form many contacts in different orientations for minimizing repulsive interactions with the substrate and previously adsorbed proteins (Rusmini et al., 2007), however, by its weakness, reversible binding could usually occur (A. Sassolas, Blum, & Leca-Bouvier, 2011).

Ocana *et al.* (2012) developed a reusable impedimetric aptasensor for the detection of thrombin in which the immobilization of the aptamer onto the transducer surface was conducted by a wet physical adsorption procedure.

1.4.2. Covalent immobilization

DNA immobilization by covalent attachment is often used (Audrey Sassolas et al., 2008) and preferred over adsorption (A. Sassolas et al., 2011). Thiol-metal interactions are frequently used to bind biomolecules covalently onto gold surfaces (Bai et al., 2008; Y. S. Kim et al., 2008). Noble metal surfaces display strong affinity to thiol groups and consequently enable the formation of covalent bonds between the sulfur and gold atoms (A. Sassolas et al., 2011).

The three most common groups used for surface covalent attachment to chemically-modified sensor surfaces are hydroxyl, amine, and carboxylic acid functional groups. The choice of this kind of surface functionalization depends on the types of terminal functional groups linked to aptamers that are available (Balamurugan et al., 2008). These groups interact with chemical groups (such as amino) through covalent binding, leading to a layer of ordered film on the sensor surface (Audrey Sassolas et al., 2008; L. Zhou et al., 2011).

1.4.3. Biocoatings/Bioaffinity

Chemical affinity reactions allow a gentle oriented immobilization of proteins, providing an important improvement over other immobilization techniques (Rusmini et al., 2007).

Avidin is a glycoprotein which contains four identical subunits with a combined mass of 67 to 68 kDa (Orita, Tomita, Harada, & Kato, 2012). The specific and strong interaction between avidin (or one of its derivatives like streptavidin) and biotin has been widely explored for surface immobilization of a number of bio-receptors, including aptamers (Balamurugan et al., 2008), and it has been used in several recent studies (L. J. Chen et al., 2011; General, Dragomirova, & Meirovitch, 2011).

Biotin- avidin interaction is one of the ideal methods for aptamer fixation on a variety of sensor surfaces, as its interaction exhibits the highest known affinity in nature ($K_d = 10^{15}\ M^{-1}$), robustness and simplicity (Balamurugan et al., 2008; Orita et al., 2012; A. Sassolas et al., 2011). Specifically, compared with other fixation methods, each avidin molecule can bind with four biotinylated aptamers as shown in Figure 5. This increases the amount of aptamers potentially bound to the sensor surface, reduces the incidence of non-specific adsorption, and improves sensor signal-to-noise ratio (Zhou et al., 2011).

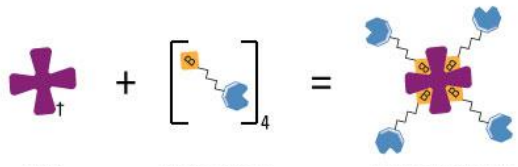

Figure 5 Illustration of Avidin-biotin interaction. Each Avidin molecule can bind four biotinylated aptamers. Taken from http://www.piercenet.com/media/Avidin-Biotin-Interaction1.jpg

Avidin-biotin method was used by Minunni and collaborators (2004) to activate a gold film-coated quartz chip using 11-mercaptoundecanol and carboxylated dextran to interact with avidin using 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide sulfosuccinimide (EDC/NHS). Afterwards, the biotin-labeled aptamers were attached to the avidin-modified quartz chip. The sensor can be used to detect HIV-1 transactivator protein, being the linear detection range between 0–2.5 mg L^{-1}, and the detection limit around 0.65 mg L^{-1} (Minunni, Tombelli, Gullotto, Luzi, & Mascini, 2004). In Hianik et al., (2007) it was used the avidin-biotin method for aptamer immobilization, in which quartz crystal microbalance (QCM) was the method used for mass detection to study the interaction of thrombin with DNA aptamers exhibiting two different configurations. The authors suggested that immobilization of the aptamer by means of avidin-biotin methodology provides the best results in sensitivity comparing to other immobilization methods using dendrimers or immobilization via chemisorption of the aptamer onto a gold surface (Hianik, Ostatna, Sonlajtnerova, & Grman, 2007).

The avidin–biotin conjugation methods possess several advantages, for example they do not depend on the isoelectric point of the protein. Nevertheless, the use of avidin/biotin immunoassays is currently limited because of their sensitivity to high temperature and organic solvents (Orita et al., 2012).

1.5. Design Strategies for Aptasensors

A sensitive and simple method to use aptamers as recognition elements for the development of biosensors (aptasensors) is to transduce to an electrochemical signal the real-time biological phenomena in solution, or through aptamer immobilization onto a solid support (Hong et al., 2012; Minunni et al., 2004; O'Sullivan, 2002). As sensing probes, aptamers are extremely advantageous in a biosensor. In fact, they can be modified for immobilization purposes and can incorporate particular reporters, without influencing their affinity, which has been very helpful for a number of design methods (Balamurugan et al., 2008; Phillips et al., 2009). Regarding the design strategies for most of the electrochemical aptasensors, four broad classes can be defined depending on the assay format and the method of detection: (a) sandwich mode; (b) label-free aptamer sensor-using electrochemical impedance spectroscopy (EIS); (c) aptamer conformational changes; and (d) target-induced displacement mode (TID), as illustrated in Figure 6 (Abe, Yoshida, & Ikebukuro, 2013; Han et al., 2010; Y. Huang et al., 2012; A.-E. Radi, 2011; Audrey Sassolas, Blum, & Leca-Bouvier, 2009; Y. Xu, Cheng, He, & Fang, 2009).

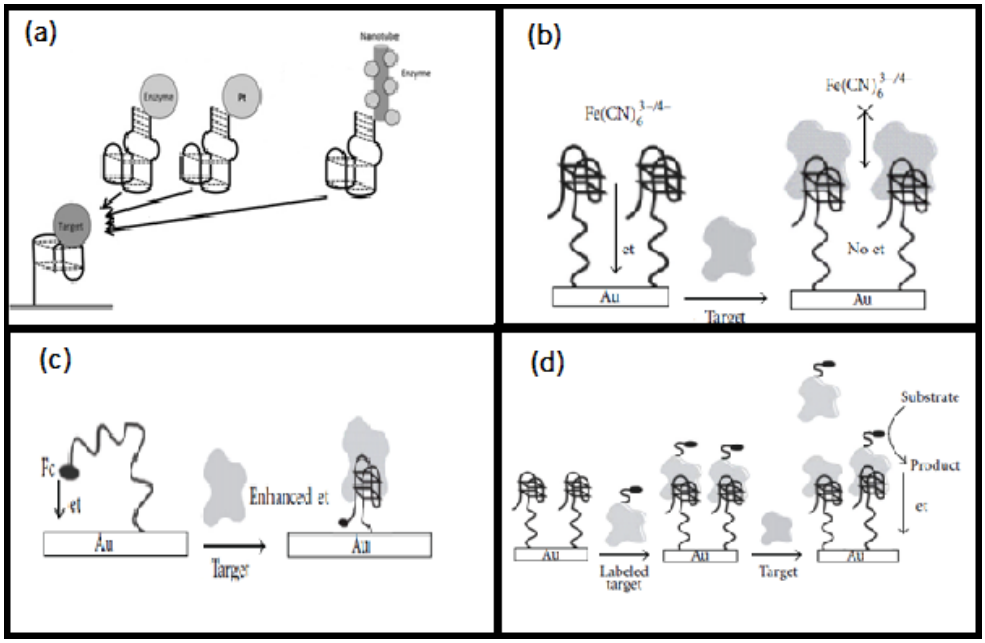

Figure 6 The schematic representation of protein strategies for electrochemical biosensors: (a) Sandwich assay using aptamers combining various signal amplification methods: enzyme, nanoparticle and carbon nanotubes means of measuring the concentration of bound target via impedance spectroscopy; (b) Impedimetric aptasensor: the binding of a target to the aptamer reduces the electron transfer (et) of a small redox mediator diffusing to the electrode surface and the increase the interfacial resistance, which provides a means of measuring the concentration of bound target via impedance spectroscopy; (c) Aptamer Conformational Changes: in the absence of target, the aptamer is largely unfolded, allowing for frequent collisions between the terminal redox moiety and the electrode. After target binding, the aptamer folds, enhancing electron transfer and producing a signal-on aptasensor; (d) Displacement Assays: target molecules in a sample displace labeled-target molecules previously bound to the sensor surface. Adapted from: Radi et al., 2011 and Abe, K., Yoshida, W., & Ikebukuro, K., 2013

1.5.1. Sandwich Assay (SA)

The sandwich structure strategy presents several advantages as compared to the strategy that uses only one recognition element to capture and label the target biomolecules. These advantages include its high sensitivity and simple operation in what regards the biosensor fabrication for biosensor fabrication. In a sandwich structured biosensor, the sequence is firstly immobilized on the electrode to hybridize with a certain part of the target DNA, and then the probing sequence is available to react with another part of the target sequence. The electroactive label at the probing sequence thus electrochemically transduces the presence/binding of the target sequence.

Therefore, as a strategy employed for aptasensor in sandwich manner, the target should have two or more recognition elements, namely the one that binds to the aptamer, used as capturing element to be immobilized on the electrode surface and catch target molecules; and the other one that serves as a probing element to mark the target with electroactive molecules or nanoparticles (Y. Xu et al., 2009).

To construct highly sensitive detection systems, some researchers have attempted the conjugation of nanoparticles (NPs), enzymes, quantum-dots (QDs) and carbon nanotubes (CNT) with aptamers (Abe et al., 2013).

1.5.2. Label-Free Aptamer Sensor -Using Electrochemical Impedance Spectroscopy (EIS)

Electrochemical impedance spectroscopy (EIS) has been mainly applied to study corrosion systems, specifically to monitor the resistance components of the impedance response (Grieshaber et al., 2008; Y. Xu et al., 2009). Therefore, it reflects changes in a diffusion-limited electrochemical process, presumably due to steric hindrance created by the bound molecules to the immobilized aptamer and repulsion between electron mediators and immobilized molecules (Abe et al., 2013).

1.5.3. Aptamer Conformational Changes

In this detection strategy, the DNA ligands recognize their targets primarily by their special shape and not by their sequence. They are capable to fold into unique complex intramolecular secondary and tertiary structures, binding and crosslinking the target molecule in a large surface area. Therefore, they are able to distinguish a small structural change in the target molecules. Besides, aptamers change its conformation through Watson-Crick base pairing and drastically, in some cases, they reduce the electronic transfer distance of the electroactive probing group from the

aptamer sequence end to the electrode surface, or desorb the electroactive intercalators from the aptamer bases, consequently resulting in a change in the electrochemical current (Abe et al., 2013). This approach based on the shift of the aptamer structure has the advantage of a readily regeneration by varying temperature or ionic strength (Y. Xu et al., 2009).

The changes of electrochemical features after target binding can be correlated to the target concentration. Signal-on (positive readout signal) and less sensitive signal-off (negative readout signal) aptasensors have been described, based on electrochemical signal producing design and signal amplifications methods, namely target binding-induced conformational change of aptamers, or on target binding-induced strand displacement, or even on both processes (Audrey Sassolas et al., 2009). "Signal-on" and "signal-off" biosensor functioning are described in Figure 7.

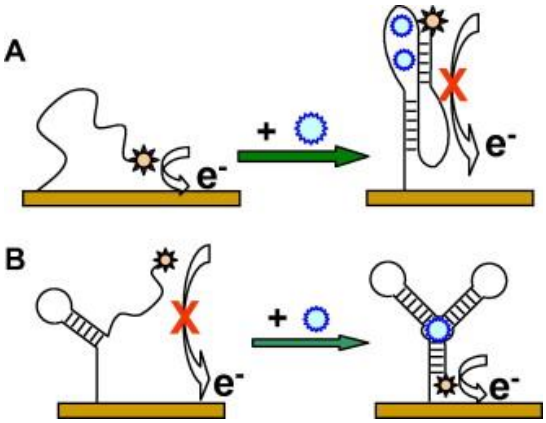

Figure 7 Signal-off (A) and signal-on (B) approaches using an aptamer labeled with a redox active molecule. Taken from de-los-Santos-Álvarez et al., 2008

The "signal-off" sensor strategy corresponds to the situations in which target recognition is signaled by the loss of an initially high current. An inconvenient of such functioning is that it limits the signal gain: one can never suppress more than 100% of the original current. Conversely, "signal-on" sensors, in theory, can provide an enormous signal gain as the background observed in the absence of target is pushed towards zero (Daniels & Pourmanda, 2007).

1.5.4. Target-Induced Displacement Mode (TID)

In the TID mode, the complementary sequences of aptamers, instead of the aptamers themselves, are used as anchors to localize the aptamers. After incubation with targets, the formed target-aptamer complexes will be released into the solution, which leads to changes of detectable signals (Han et al., 2010).

1.5.5. Other electrochemical strategies

A direct labeling method has also been used to monitor the presence of target proteins on the electrode surface. Labels such as enzymes, metal nanoparticles (NPs), other redox species

such as methylene blue (MB), ferrocene (Fc), potassium ferrocianate ($K_3Fe(CN)_6$) or anthraquinone have been used (Audrey Sassolas et al., 2009; K. M. Song et al., 2012; Beate Strehlitz et al., 2008). Frequently, the amount of the labeled molecules indicates the amount of DNA targets in the sample, either in a direct detecting or a competitive detecting mode (F. Wei et al., 2010). These labels are commonly covalently linked to the terminal groups of aptamers.

The labeled position has to be carefully selected so it does not interfere with the folding of the aptamer, or to guarantee that it does not modify an essential binding group. If labeling is incorrectly performed it can drastically change the anticipated binding properties, and the yield of the target-label coupling reaction will be extremely variable. Therefore, an indirect labeling scheme often referred to as a sandwich assay is commonly used for protein detection. This requires two probes that bind to different regions of the target, yielding enhanced selectivity but increasing development costs and limiting its use in research settings (Daniels & Pourmanda, 2007).

Table 4 presents a comparison between the properties of several electrochemical aptasensors against thrombin.

Table 4 The comparison of the representative electrochemical aptasensor using different detection schemes. Adapted from: Hamula et al., 2006; Kim et al., 2008; Sassolas et al., 2009; Xu et al., 2009; Radi, 2011 and Abe, 2013

Aptasensing Strategy		Material	Detection Limit	Reference
Sandwich structure	Marking enzymes on probing recognition element	GDH	10 nM (thrombin)	(Ikebukuro et al., 2005)
	Nanoparticles on probing recognition element	Au NPs	0.1ng/mL	(He, Shen, Cao, & Li, 2007)
	Magnetic beads coated with streptavidin	StreptAvidin magnetic beads	0.45 nM (thrombin)	(Centi et al., 2007)
	Marking electroactive molecules/ nanoparticles on probing recognition element	CdS particles	0.14 nM (thrombin)	(Numnuam et al., 2008)
	Screen-printed carbon electrode first aptamer immobilization(SPCE) and Au NPs second aptamer immobilization	Au NPs	1 nM (thrombin)	Look up at (A.-E. Radi, 2011)

Aptasensing Strategy		Material	Detection Limit	Reference
EIS	Change at electrode R_{ct}	Interference of protein to $[Fe(CN)_6]^{3-/4-}$	2 nM (thrombin)	(A. E. Radi, Acero Sanchez, Baldrich, & O'Sullivan, 2005)
	Protein binding event via monitoring the interfacial electron transfer resistance with EIS	Interference of protein to $[Fe(CN)_6]^{3-/4-}$	0.1 nM (thrombin)	(Cai, Lee, & Hsing, 2006)
	Protein binding event via monitoring the interfacial electron transfer resistance with EIS	Guanidine hydrochloride	$1.0x10^{-14}$ mol L^{-1} (thrombin)	(Y. Xu, Yang, Ye, He, & Fang, 2006)
	Physical and electrical properties of carbon thin film pyrolyzed with a positive photoresist	Pyrolyzed carbon	0.5nM- 500nM (thrombin)	(J. A. Lee et al., 2007)
	Molecularly imprinted polymers	Methylene green (MG)	0.5 nM (thrombin)	(Evtugyn et al., 2008)
	Changes of the interfacial properties of the electrode	Graphite-epoxy composite electrode (with $[Fe(CN)_6]^{3-/4-}$)	4.5nM (thrombin)	(Ocana, Pacios, & del Valle, 2012)
Aptamer Conformational Changes	Signal-Off	Releasing intercalated MB	11 nM (thrombin)	(Bang et al., 2005)
	Signal-On	MB labeling	6.4 nM (thrombin)	(Xiao, Lubin, Heeger, & Plaxco, 2005)
	Chronoamperometric beacon system	Fc-labeled	3.5nM (thrombin)	(Mir, Vreeke, & Katakis, 2006)
	Signal-On	Pushing labeled Fc close to electrode	0.5 nM (thrombin)	(A. E. Radi, Acero Sanchez, Baldrich, & O'Sullivan, 2006)
	Switching structures of aptamers from DNA/DNA duplex to DNA/target complex	MB labeling	3 nM (thrombin)	(Yan, Wang, & Chen, 2011)
Target Molecule Displacement	Displacing probing protein from electrode	Quantum dots tagged Probing proteins	0.5 pM (thrombin)	(Hansen et al., 2006)
	Displacing complementary sequence from electrode	Fc-labeled complementary sequence	<2 nM (thrombin)	(Lu, Zhu, Yu, & Mao, 2008)
	Displacing CdS-CDs with sequence from the electrode	CdS nanoparticle surface-modified *B*-cyclodextrins (CdS-CDs)	$4.6x10^{-12}$ M (thrombin)	(Fan, Li, Wang, He, & Fang, 2012)
	Displacing Fc-AuNPs from electrode	Ferrocene-coated gold nanoparticles (Fc-AuNPs)	100 pM (alfa- thrombin)	(Kwon, Jeong, & Chung, 2011)

Aptasensing Strategy		Material	Detection Limit	Reference
Signal Amplification Using Sandwich Assay Format	Amplification ability of Au nanoparticles carrying multiplex CdS NPs	Au NPs with CdS NPs	$5.5x10^{-16}$ M (thrombin)	(Ding, Ge, & Lin, 2010)
Other Electrochemical Aptasensor	Direct labeling method	MB labeling	10 nM (thrombin)	(Hianik, Ostatna, Zajacova, Stoikova, & Evtugyn, 2005)
	EQCM	Mass increase	0.3 nM (thrombin)	in (Y. Xu et al., 2009)
	Potential change	Potential change at aptamer electrode	1 nM (thrombin)	In (Y. Xu et al., 2009)

1.6. Biosensor parameter criteria

Although the development of clinical sensors and biosensors has increased in recent years, improvements in sensitivity, selectivity, limits of detection, fast response and miniaturization are yet to be achieved. Health care appears to provide the best opportunity for sensor development.

Electrochemical biosensors are the most common in the clinical field, due to their high sensitivity and selectivity, portability, rapid response time and low cost.

The characterization of a biosensor response is important since the sensor operating parameters will dictate its performance and further optimizations (Justino, Rocha-Santos, & Duarte, 2010) (Thévenot, Toth, Durst, & Wilson, 2001). The fast spread of the biosensors field led to the actual situation in which performance criteria are still not well defined. Thus, standard IUPAC definitions will be followed, in order to establish the guidelines for reporting characteristics of a biosensor response (Thevenot, Toth, Durst, & Wilson, 1999).

Several parameters should be discussed in order to fully characterize a biosensor such as calibration, sensitivity, limit of detection (LOD), selectivity, steady-state responses times and concepts concerning the final results, i.e., reproducibility, reliability, stability and lifetime for assessing analytical accuracy (Justino et al., 2010; Thevenot et al., 1999).

Sensor calibration is the first point to evaluate. This calibration is performed using standard solutions of the analyte and plotting steady-state responses *versus* analyte concentrations. Steady-state response time refers to the time necessary to reach 90% of the saturation (Thévenot *et al.*, 1999). Within the linear range of response for the method, the sensitivity is a well-defined value. If a small change in the analyte concentration causes a large change in the response it can be concluded that the analytical method is sensitive (Justino et al., 2010). Regarding the working concentration range, the LOD corresponds to the concentration at which the smallest signal can be detected with an acceptable degree of certainty for a given analytical procedure (Justino et al., 2010). Additionally, the biosensor selectivity is expressed as the ratio of the signal output with the analyte alone to that with the interfering substance alone, both at the same concentration. Consequently, the response of the analyte of interest can be differentiated from other responses.

Reproducibility is a measure of the discrepancy on a series of observations or results performed over a period of time. Usually, it is determined for the analyte concentrations within the usable range of the biosensor. Reliability is also crucial for the biosensor precision. It depends both on the selectivity and reproducibility of results. A biosensor is reliable when the response is directly

related to the analyte concentration and it does not vary with concentration fluctuations of interfering substances within the sample matrix (Justino et al., 2010; Thévenot et al., 2001).

Depending on the sensor geometry, method of preparation, as well as on the receptor and transducer used, the working stability of a biosensor response will be altered. These parameters are intrinsically dependent upon the response rate-limiting factor, i.e. by the biological recognition reaction. Hence, to eliminate this type of variations, experimental conditions such as pH, ionic strength, temperature, analyte concentration, and presence of organic solvents, as well as sample size and sample pre-treatment have to be established *a priori*. Furthermore, to have a good storage stability there are a number of variables that must be considered including the dry/wet atmosphere composition, pH, buffer composition and presence of additives (Labuda et al., 2010; Thevenot et al., 1999). Finally, the characterization of the biosensor parameters is also essential for the assessment of the sensor lifetime.

2. Materials and methods

2.1. Fluorescence anisotropy assays

Fluorescence is a technique commonly used to measure binding events, and the fluorescence recognition reaction does not require the separation of the blank reagent (control) from the test media (Gokulrangan et al., 2005). With the recent advancements in synthesis of fluorochrome-labeled oligonucleotides and the advent of more sophisticated fluorescent plate-readers, fluorescence offers an excellent methodology for determining binding affinities between different species. A significant anisotropy change will be observed when an aptamer labeled with a fluorescent dye (e.g. fluorescein) binds to the target molecule (e.g. protein). By placing the fluorescent signal on the smaller DNA molecule, binding to the much larger protein results in a substantial change in anisotropy, and therefore enables the determination of binding constants within the range of 10^{-10} to 10^{-3} M (Owen & McMurray, 2009) (Figure 8).

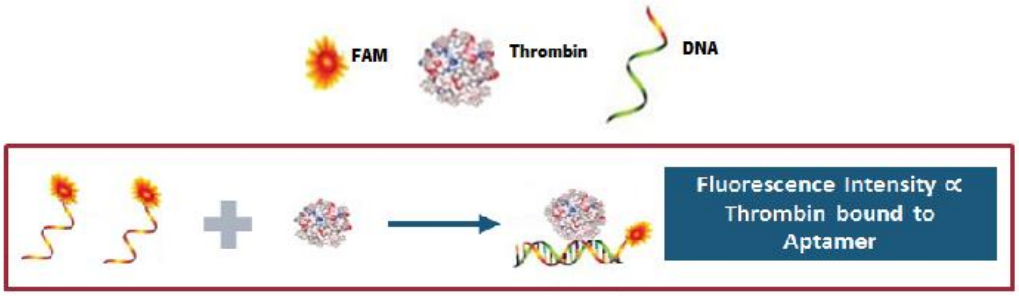

Figure 8 Schematic of DNA aptamer binding to thrombin. Taken from: http://www.piercenet.com/method/Avidin-biotin-interaction)

Several label-based or label-free techniques have been demonstrated to achieve the reliable detection of proteins, including the enzyme-immunosorbent assay (ELISA). Combining real-time measurements with sensitivity, selectivity and reversibility it is interesting to monitor individual binding events (S. Liu et al., 2011).

2.1.1. Materials and chemicals

Thrombin-binding DNA sequences used in the current work were designated unmodified 15-mer Thrombin binding aptamer 1 (TBA1): 5'-GGT TGG TGT GGT TGG-3', unmodified 26-mer Thrombin binding aptamer 2 (TBA2) : 5'-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3' and Osteopontin-binding RNA sequence (OPN-R3): 5'-

CGGCCACAGAAUGAAAAACCUCAUCGAUGUUGCAUAGUUG-3' (Table 5). In all aptamers, the fluorophore 6-carboxyfluorescein was attached to the 5`-end of the DNA and RNA (in the case of the OPN-R3) to enable their detection by the fluorescence anisotropy assay. All the aptamers (thrombin and osteopontin) were purchased from IDT Integrated DNA Technologies (Munich, Germany). The stocks were dissolved in deionized purified MilliQ water, pH 6, to a final concentration of 100 μM, and from this volume several aliquots were stored at −20°C.

Table 5 List of the DNA and RNA sequences used in the experiments

Designation	Sequence
Labeled unmodified 15-mer Thrombin binding aptamer 1 (TBA1)	5`- 6FAM- GGTTGGTGTGGTTGG-3`
Labeled unmodified 26-mer Thrombin binding aptamer 2 (TBA2)	5`- 6FAM- AGTCCGTGGTAGGGCAGGTTGGGGTG-3`
Labeled modified Osteopontin aptamer (FAMR3-OPN)	5'-6FAM-CGGCCACAGAAUGAAAAACCUCAUCGAUGUUGCAUAGUUG-3'.

Thrombin from human plasma (T6884) was purchased from Sigma-Aldrich (Saint Louis, MO, USA) and human osteopontin (hopn, catalog N. 1433-OP/CF) was purchased from R & D Systems (Minneapolis, MN 55413, USA). Other proteins such as Bovine serum albumin (BSA) and Bovine plasma osteopontin (bopn) were bought from Sigma Chemical Co. (St Louis, MO, USA) and from R & D Systems (Minneapolis, MN 55413, USA), respectively. All proteins were dissolved in deionized purified (MilliQ) water and proper aliquots were stored according to the supplier's instructions.

All other chemicals used in the current work were of analytical grade and used without further purification. Deionized water was obtained using an ultrapure water system Millipore (ELIX5. USA) and had an electrical resistance larger than 18.2 MV.

2.1.2. Fluorescence apparatus

The method used to evaluate the aptamers fluorescence was described by Li *et al.,* 2007 (W. Li, Wang, Tan, Ma, & Yang, 2007). Briefly, fluorescence measurements were performed in 96-dark well plates using an ELISA (Synergy HT, BIO-TEK, IZASA) equipped with a thermostat holding a temperature control accuracy of 0.1˚C. All experiments were carried out at 25˚C. The fluorescence of the aptamers was monitored by exciting the sample (6-FAM-labeled) at 492 nm and measuring the emission at 518 nm. The Gen5 software was used. This software is capable of collecting

microplate data and conduct analysis after settle a range of excitation and emission wavelengths for the test.

Protein samples were prepared individually in aluminum-coated eppendorfs containing 1X Phosphate Buffer Solution (PBS) pH= 7.6 freshly prepared. The use of PBS buffer enables a constant pH and osmolarity, but also ion concentrations in the samples similar to ones in the human body (isotonic). Moreover, using a buffer solution also prevents denaturation or other conformational changes in aptamer and protein samples. The 10X PBS was prepared dissolving 80 g of NaCl, 2 g of KCl, 14.24 g of $Na_2HPO_4.2H_20$, 2 g KH_2PO_4 in 800 mL distilled H_2O. After adjusting the volume to 1 L with ultra-pure water, the PBS was autoclaved and stored at room temperature. Before the fluorescence measurements, 100 mL of fresh 1XPBS was prepared from the stock solution (10XPBS) and immediately used to prepare the protein samples.

A 60 µl volume of purified proteins at increasing concentrations (0, 3, 10, 30, 100, 300, 350 nM) was added to the 96-dark well plates, as well as 20 nM of fluorescein-labeled DNA/RNA (aptamer). The mixture was incubated at 25°C for 30 min in the dark (to avoid loss of fluorescence and interferences with the assay). Figure 9 illustrates the scheme of the samples in the 96-well plate for the fluorescence assays.

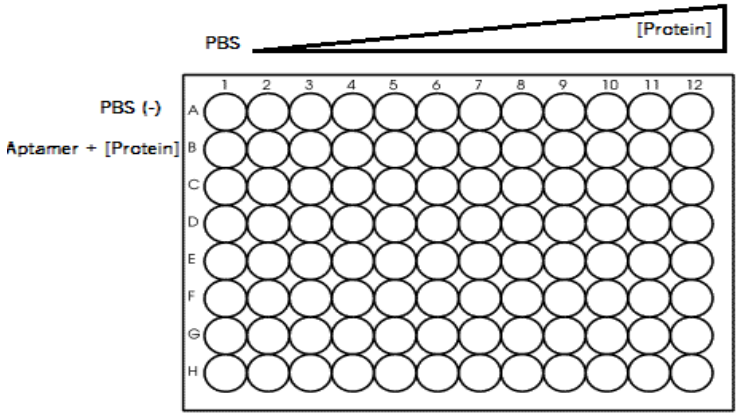

Figure 9 Scheme of the 96-dark well plate for the fluorescence measurements

Prior to incubation with proteins (human thrombin and osteopontin, and BSA (interferent)), the aptamer samples) were denatured at 95°C for 5 minutes to unfold the DNA/RNA strand. Then, they were cooled in ice for 5 minutes to block the DNA/RNA in its unfolded structure. Finally, the aptamers were left to cool down to room temperature (RT) for a residual slow annealing (Tran et al., 2010; Zou et al., 2012).

Since during the course of the current work it was not possible to perform the fluorescence measurements in triplicate, mainly due to the amounts of samples that were available and to the costs of the proteins being studied, in order to compare results obtained with proteins from the same preparation mode (same batch), the same 96-dark well plate that was used to read the samples after 30 minutes of reaction, was reused to check the binding reaction after 1 and 4 hours.

2.1.3. Fluorescence anisotropic measurements and dissociation constant (K_d) data fitting

Due to the experimental difficulty in measuring the amount of free DNA aptamer, it is crucial that K_d is accurately determined through the use of the total DNA amount in the calculations. At large ratios of aptamer to ligand concentration, the total concentration of the ligand can be regarded as sufficiently small to be ignored.

The fluorescence data along time were converted into aptamer:protein fluorescence and afterwards into concentrations. A non-linear fitting was conducted using the solver tool from excel in order to determine the K_d for TBA1 and TBA2.

The set of equations (1 to 5) described below were used for the K_d determination. These involve the following parameters: F_T, total fluorescence; F_{APTNB}, fluorescence of the aptamer not bound; and $F_{COMPLEX}$, fluorescence of the complex. As the one-to-one binding stoichiometry for aptamer binding to a protein can be written as:

$$F_T = F_{APTNB} + F_{COMPLEX} \quad (1)$$

$$F_T = a + b[APTNB]_i + c[COMPLEX]_i \quad (2)$$

$$F_T = a + b\{[APTNB]_0 - [APTBOUND]_i\} + c[COMPLEX]_i \quad (3)$$

$$F_T = a + b[APTBOUND]_0 - b\,[COMPLEX]_i + c[COMPLEX]_i \quad (4)$$

As in the equilibrium the total amount of DNA should be bound to protein, [APTBOUND] ≈ [COMPLEX], as a result,

$$[\boldsymbol{COMPLEX}]_{\boldsymbol{i}} = \frac{\boldsymbol{F_T - a - b[APTNB]_0}}{\boldsymbol{c - b}} \quad (5)$$

where, “a” is null (“a” is the correction parameter applied in all fluorescence values when a null protein concentration is measured. In an attempted to determine this value it was assumed that the fluorescence data were corrected and related with the calibration curve of the aptamer involved); “b” is the coefficient between the emitted fluorescence and the aptamer concentration used in the experiment (only by the aptamer, i.e. when the protein concentration is null); and “c” is the fluorescence value that corresponds to an aptamer concentration of 20 nM. Several assays

were performed, in order to establish 20 nM as the final concentration of aptamer. However, the concentration range studied took into account studies carried by other authors (Gokulrangan et al., 2005).

2.2. Electrophoretic mobility shift assays (EMSA)

The electrophoretic mobility shift assay (EMSA) is one of the most sensitive methods for studying the DNA-binding properties of a protein. This method can be used to deduce the binding parameters and relative affinities of a protein for one or more DNA sites, or for comparing the affinities of different proteins for the same sites. The protein-DNA complexes are separated from free (unbound) DNA by electrophoresis through a non-denaturing polyacrylamide gel.

This method relies on the property that nucleic acid will migrate through an agarose or polyacrylamide gel matrix towards an anode upon application of an electric field. Through this approach, the protein retards the mobility of the DNA fragments to which it binds. Thus, the free DNA migrates faster through the gel than the DNA protein complex (Sosic et al., 2011) (Figure 10).

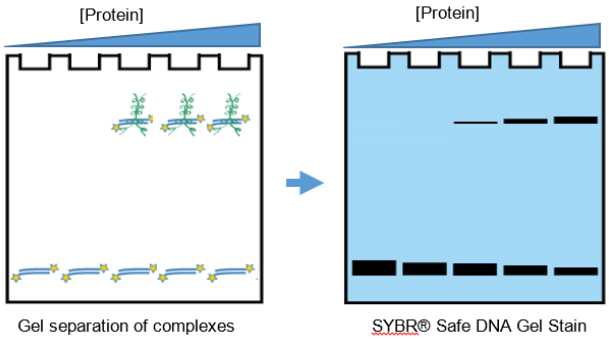

Figure 10 Electrophoretic mobility shift assay representation. The gel shift assay consists of three key steps: binding reactions, electrophoresis and probe detection. Adapted from http://www.piercenet.com/media/EMSAOverview615x416.jpg.

In a somewhat simplified view, the migration of DNA through a gel is governed by three primary factors, the molecular weight (and hence the charge) of the DNA, its three-dimensional shape, and the physical properties of the gel substrate (Ryder, Recht, & Williamson, 2008). In the study from Sosic and co-workers (2011), EMSA was performed to verify whether the chemical modifications introduced in TBA1 and TBA2 would affect aptamer/protein recognition. The results showed that the band corresponding to unmodified TBA2 aptamer in KCl 10 mM folds in G-quadruplex shifts up and new bands with lower mobility, representing the TBA2-thrombin complex, appear in the upper part of the gel, as expected.

2.2.1. Reagents

TEMED (N,N,N′,N′-Tetramethylethylenediamine) (Sigma-Aldrich) was stored tightly sealed at 4°C. Ammonium persulfate (APS) (Sigma-Aldrich) was stored in a desiccator at 4°C. APS was used in a 10% w/w solution to be used as a catalyst for the copolymerization of acrylamide and bisacrylamide gels. The polymerization reaction was driven by free radicals generated by a redox reaction in which TEMED (a diamine) was used as the adjunct catalyst. A 40% w/v acrylamide-bisacrylamide solution for electrophoresis contain the proportion 37.5:1 of acrylamide: bisacrylamide (BIO-RAD) was prepared and kept at 4°C and protected from light until use. All reagents were purchased by Sigma-Aldrich.

Tris-borate-EDTA (TBE) buffer is often used for gel electrophoresis in the analysis of DNA reactions. For the experiments, TBE 5X buffer was prepared, dissolving both 54 g Tris base and 27.5 g boric acid (both from Sigma-Aldrich) in approximately 900 mL deionized water. Afterwards, 20 mL of 0.5 M EDTA (pH 8.0) was added and the volume solution was adjusted to 1 L. Then TBE 5X was stored at RT and used until precipitate was formed. Deionized purified (MILLIPORE, ELIX5) water was also required for the experiment and all the other reagents used were from acceptable molecular biology grade. Commercial petroleum jelly was used to seal the plates before the gel apparatus was assembled.

All the complexes resultant from the ELISA assay were also used for the EMSA experiments as well.

2.2.2. Equipment

An electrophoresis power supply and a vertical electrophoresis apparatus were used. This apparatus includes glass plate spacers (1.0mm), well-forming combs, clamps and optionally, a gel-casting stand.

2.2.3. EMSA Analysis for Binding Complexes

After 4 hours of protein and aptamer mixture incubation, a gel electrophoresis was carried out to confirm the formation of the complex protein:aptamer. The methodology used was adapted from Sosic *et al.* 2011 (Sosic et al., 2011). Briefly, the protein-aptamer complexes were resolved by a 12% non-denaturing acrylamide gel with 4% stacking-gel. The gels were prepared from the solutions described in Table 6.

Table 6 Solutions for preparing resolving gels for NATIVE-PAGE gels

12% Resolving Gel (for two gels)			4% Stacking Gel (for two gels)	
	% (v/v)	mL	% (v/v)	mL
Acrylamide Mix	40	3	40	0.225
Glycerol	50	0.2	-	-
APS	10	0.075	10	0.03
TBE 5X Buffer		1		0.6
TEMED		0.00075		0.0003
Water		5.2775		1.575
Total Volume of Gel		5		3

(Note: It is important that the gel solution (without TEMED) must be stirred at room temperature, under vacuum, until all bubbles are eliminated. The gel polymerization can then proceed by adding TEMED).

While gels are polymerizing, samples from the 96-dark well-plate were collected. Samples were individually prepared in appropriate volumes, by pipetting 10 µL of each protein-aptamer mixture (from the previously ELISA described binding reactions) and add with 10 µL of bromophenol blue dye.

After polymerizing, the 12% non-denaturing polyacrylamide gels were placed in the electrophoresis apparatus. TBE 1X was added to the top and bottom reservoirs. After removing all the bubbles that were trapped, 20 µL of each samples were loaded. Also, a 100 kb DNA marker (New England Biolabs) was used. Prior to loading, the marker was denatured at 95°C for 5 minutes and was cooled for 5 minutes extra to allow the visualization of the bands by naked eye.

The electrophoresis was carried at 200 V constant voltage for about 1hour and 45 minutes. After running the gels, aptamer-protein complexes were stained with the fluorescent DNA binding dye Sybr®Safe (Invitrogen) (5 µl/ 50 ml TBE 1X) for 10 minutes at RT and the results documented by exposure of the dried gel to Chemidoc program (BioRad).

2.3. Immobilization Assay on Screen-Printed Electrode

2.3.1. Apparatus

Electrochemical analysis was performed at room temperature (RT) using an electrochemical analyzer PGSTAT-10 Autolab (Ecochemie). The screen-printed gold electrode (SPE) was composed of a working, reference and counter electrode. The working electrode had a diameter of 0.8mm (DropSens, S.L., Spain).

2.3.2. Reagents and solutions

Thrombin-binding DNA sequences entitled as unmodified 15-mer thrombin binding aptamer 1 (TBA1): 5'-GGT TGG TGT GGT TGG-3' and unmodified 29-mer thrombin binding aptamer 2 (TBA2) : 5'-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3'. Both aptamers have been designed to possess in the 5`end a biotin molecule, since this is a typical motif with high affinity to avidin that will be used to functionalize the surface of the electrode (immobilization step). The aptamers were synthesized by IDT Integrated DNA Technologies (Munich, Germany).

The protein used was of analytical grade highly purified. Thrombin from human plasma (T6884) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Thrombin samples were prepared in PBS at pH 7.4 (Z. Liu et al., 2009).Thrombin samples were prepared from a 20 μM aliquot at different concentrations (0.025, 0.05, 0.5, 1 and 50 nM).

Regarding the reagents used, the 3,3`-dithiopropionic acid-di(N-succinimidylester) (DSP) was purchased from Fluka and used in a 1 mg/mL concentration. Dimethyl sulfoxide (DMSO) required for the preparation of DSP was purchased from (> 99%, Fluka). The PBS stock solution (0.1M) was prepared as described in the previous assays.

As described in Wu *et al.,* 2011 (Wu, Zheng, Shen, & Yu, 2009), the redox solution was prepared with a final concentration of 5mM $K_4[Fe(CN)_6]$:$K_3[Fe(CN)_6]$ in 100 mM. The following amounts were weighed, 215 mg of $Fe(CN)_6]^{4-}$, 165 mg of $Fe(CN)_6]^{3-}$ (both from Acròs Organics) and 75 mg of KCl (Panreac), and further dissolved in 100 mL of PBS 1X. After homogenizing the solution, the flask covered by aluminum foil containing the redox probe was degassed in an ultrasound sonicator bath (Tesla) for 15 minutes.

In the regeneration step, 7M of urea was used (purchased from National Diagnostics).

2.3.3. Aptamer immobilization onto the gold electrode surface by avidin-biotin methodology

To immobilize the aptamer onto the gold surface, as shown in Figure 11, no cleaning step was required. The first step of the immobilization procedure was to confirm the cyclic voltammogram (CV) curve. Experimentally, the three electrodes were etched with 60 μL of redox solution $[Fe(CN)_6]^{3-/4-}$ under an optimized electric potential within -0.5 to 0.5V and a step potential of 0.05V/s. Then, the electrode was washed with PBS and left to dry at RT. Subsequently, the gold surface was modified with 1 μl of 4 mM DSP. This procedure lasted 15 minutes, RT. After washing again the electrode with PBS, the working electrode was washed three times with 1mg/mL of the avidin solution and then it was incubated at 4˚C with this protein approximately 23 hours.

After avidin incubation, the electrode was washed twice with PBS and was incubated for 1hour in BSA (0.025%) solution. This method was used to block free binding sites on the activated electrode surface. The electrode was again washed with PBS and left to dry at RT until the next immobilization step.

Before the immobilization, proper folding of the aptamer has been provided by heating it to 95˚C for 5 minutes to unfold the DNA strand. Next, the aptamer was cooled in ice for 5 minutes (to block the DNA in its unfolded structure), and finally it was left to cool to RT (for a residual slow annealing) (Tran *et al.,* 2010) (Zou et al 2011) and then cooled in ice for 10 minutes. After conditioning the electrode with the aptamer solution (1μM biotinylated-aptamer solution) for 30 minutes at RT, the electrode was again washed with PBS and it was carefully cleaned before analysis.

Thereafter, on SPE it was performed cyclic voltammetry (CV) analysis with 60 μL of the redox solution to characterize the bioaffinity immobilization of the aptamer to the gold electrode using the analyzer. All the steps involved in aptamer immobilization are described in Figure 11.

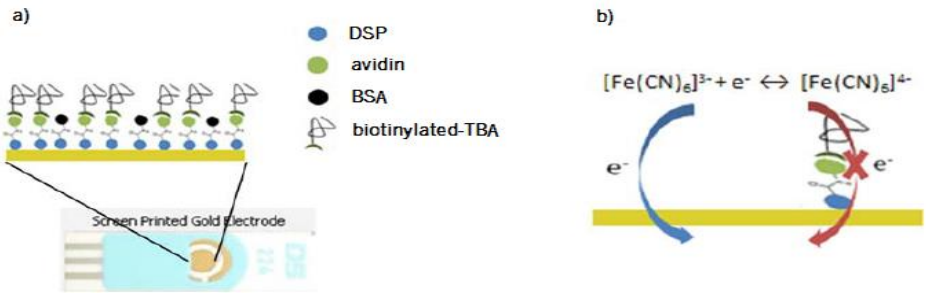

Figure 11 a) A schematic diagram of a TBA aptamer immobilized on the working electrode region of a screen-printed electrode chip. b) Scheme of signal off system through interference in the electron transfer. Adapted from Kim, Y. J. et al., 2010

It presents the detailed detection process of the electrochemical aptasensor for human thrombin. The screen-printed electrode (SPE) is first modified by a homogeneous film of DSP/avidin/BSA. This step leads to an increase of the effective surface area of the electrode and provides an enhanced loading surface for the subsequent aptamer immobilization.

Afterwards, the electrode was used immediately to measure different protein (thrombin) concentrations; otherwise it was kept at 4 °C for less than 1 week until further use (Hianik et al., 2007).

2.3.4. Experimental detection of Thrombin

Human thrombin samples were prepared in PBS 1X pH 7.4. For protein detection, 1 µL of each thrombin sample concentration was dropped on working electrode at RT for 30 minutes. After carefully washing the SPE with PBS 1X and left it to dry, 60 µL of the redox solution was placed on the sensor surface until all the three electrodes were immersed. Cyclic voltammetry was performed and the correspondent voltammograms were obtained. The experimental setup is characterized in Figure 12.

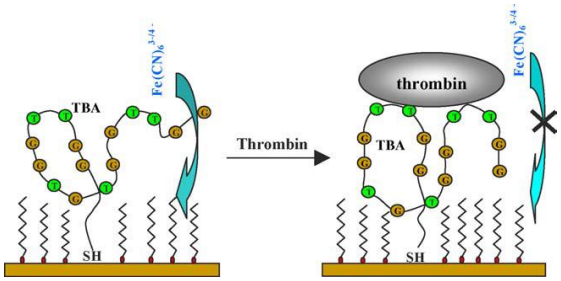

Figure 12 Electrochemical sensing of thrombin using thrombin binding aptamer (TBA) functionalized gold electrode. Taken from: Cai et al., 2006

2.3.5. Electrode sensor-surface regeneration

To dissociate thrombin and regenerate the aptamer layer, the electrode was incubated with 1 µL of 7M urea buffer and incubated for 2 minutes. Then, the SPE was washed with PBS and CV curve was performed. This process of surface regeneration should not compromise the sensitivity of the aptamer layer and should allow testing the same aptamer layer with compound concentrations of thrombin (Y. Liu, Tuleouva, Ramanculov, & Revzin, 2010).

3. Results and discussion

The results gathered in this thesis are presented and discussed in this chapter and are divided in two main sections: binding assays and development, optimization and validation of an aptasensor for the detection of thrombin.

3.1. Binding Assays

3.1.1. Fluorescence: Effect of the Labeling Strategy. Predictions & Observations.

Binding assays were conducted with 3 different fluorescein-labeled aptamers: OPN-R3FAM, TBA1FAM and TBA2FAM. OPN-R3FAM is an aptamer that has been reported to bind specifically to human osteopontin (Zhiyong Mi et al., 2009); TBA1 is a 15-mer DNA aptamer which binds exosite I of thrombin (Fibrinogen Binding Site) (Tasset, Kubik, & Steiner, 1997); while TBA2 is a 29-mer DNA aptamer binding to exosite II of thrombin (Heparin Binding Domain) (D. W. Huang et al., 2010; Sosic et al., 2011).

It is noteworthy that the 6-FAM- labeled aptamer is a relatively small molecule compared to the size of the proteins that they target. It is expected that when the aptamer binds to the protein, the increase of the overall size and molecular weight of the complex leads to a pronounced slowdown of the fluorescein molecule rotation. The slower rotational diffusion would result in a lower ability of the fluorescein molecule to depolarize the incident polarized excitation. Consequently, the anisotropy of the emission would also increase to reflect the slower motion of the dye itself.

In these assays, the 3 aptamers were incubated with four different proteins, human recombinant Osteopontin (rhOPN), bovine OPN (bOPN), serum bovine albumin (BSA) and human thrombin (hThr). According to NCBI, bOPN is known to have 55% homology with hOPN. Therefore, it is a useful compound to be used as a model to evaluate the OPN-R3FAM specificity. Further on, assays should be conducted with rhOPN to evaluate an even higher specificity of the aptamer, i.e. to confirm if it can distinguish between human and bovine OPN. Besides, Beausoleil *et al. (*2011) reported that OPN is cleaved by thrombin. Therefore, the assessment of the potential binding between OPN-R3FAM and thrombin will also enable the confirmation of the aptamer specificity. The same approach was used for TBA1 and TBA2 characterization.

3.1.1.1. ELISA`s method validation

Human OPN is a protein usually overexpressed in tumors and serum of women with ovarian and breast cancer (Rodrigues, Lopes, Sousa, Vieira, & Milanezi, 2009) and it is found in concentrations at the nanomolar level (Kadkol et al., 2006). This protein is a potential therapeutic target for the regulation of cancer metastasis, thus the RNA aptamer technology targeting OPN may represent a clinically viable diagnostic tool to validate the method for assessing the formation of aptamer-protein complexes.

In the experiments, the OPN-R3FAM aptamer concentration was fixed at 20 nM as previously established in other experiments. Moreover, since the results obtained with different incubation times showed similar profiles, the following analyses were conducted with the shorter binding reaction time, meaning 30 minutes. The binding between 20 nM of OPN-R3FAM and rhOPN was evaluated through fluorescence anisotropy analysis as illustrated in Figure 13.

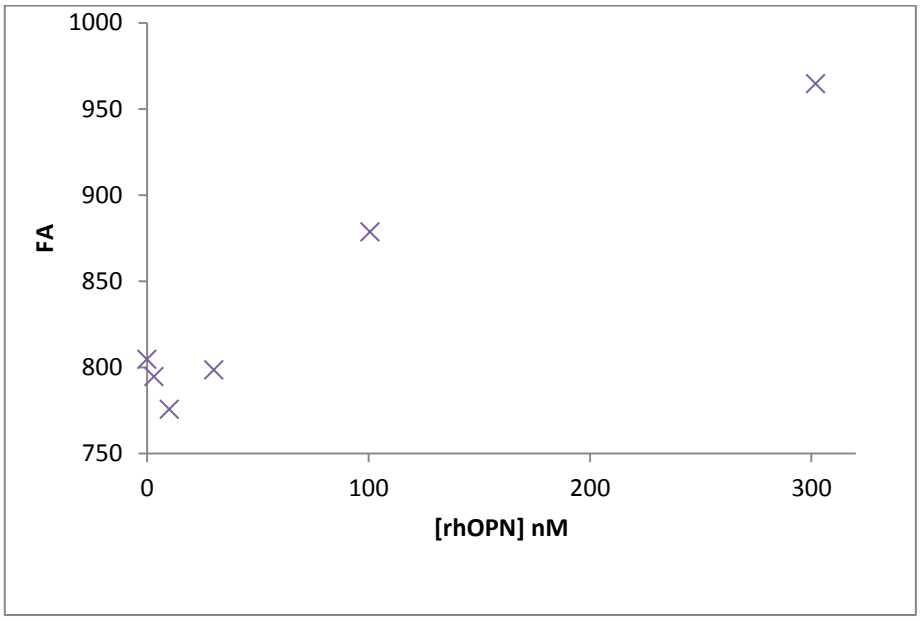

Figure 13 Single binding assay for OPN-R3FAM and hOPN. The aptamer concentration used was 20 nM, the binding reaction time was 30 min and the fluorescence was recorded at 518 nm

An appreciable increase of fluorescence anisotropy was found after incubating the protein with the aptamer, thus indicating the formation of the aptamer–rhOPN complex. After the formation of the protein-aptamer complex no further increases in the fluorescence should be observed, because all major fluorescent components are already bound to the aptamer.

The initial nonlinear fitting analysis of the fluorescence data using the binding model described in the Materials and methods section showed that the aptamer binding site on each target molecule is a one-to-one binding model. The assumption that one target molecule binds to each aptamer,

significantly improves the fit of the data, however further information on OPN will be necessary to conclude about this. The non-linear fitting of our data led to a K_d value around 5.85 nM for 30 minutes of incubation which is better than the one reported by Mi and co-workers (2009). In their study, RNA electrophoretic mobility assays were used to characterize the aptamer sequence of OPN-R3 against rhOPN, and a K_d value of 18 nmol/l was obtained. It is difficult to determine the reasons for the differences obtained for the aptamer since different methodologies were used to evaluate the formation of the aptamer-protein complex.

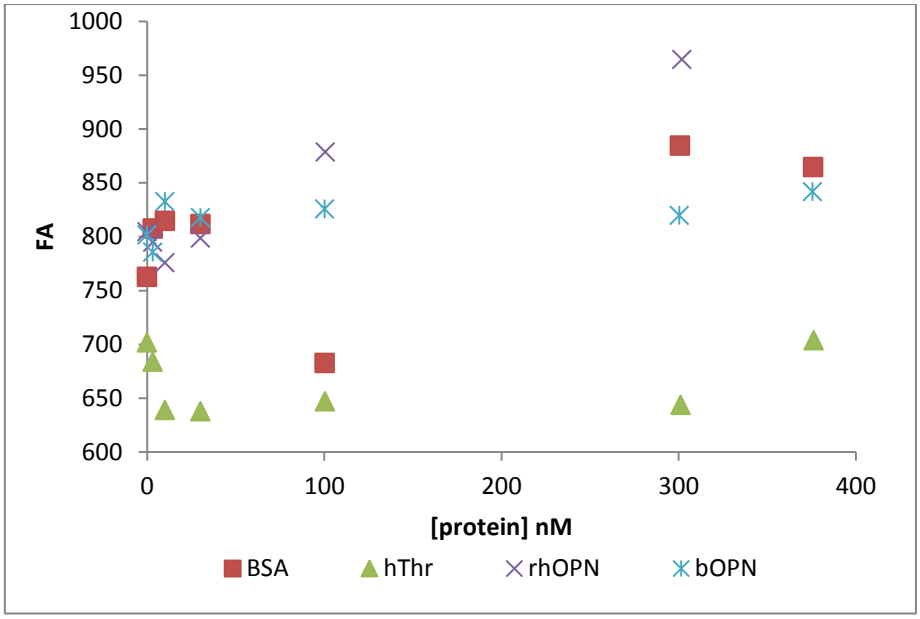

Figure 14 Plot 20nM OPN-R3FAM fluorescence responses at 528nm versus protein concentrations (bovine osteopontin, bOPN, bovine serum albumin, BSA, human blood thrombin, Thr and human osteopontin, rhOPN) in the standard phosphate buffer

A major advantage of the fluorescence assay is that it does not present interference from non-specific interactions between proteins and the aptamer probe. To assure its practical use, any protein probe should be able to distinguish between its target and other interfering proteins. While the aptamer sequence has been reported to be highly selective for OPN, it is still important to test its selectivity by using other interfering proteins in the fluorescence assay. Furthermore, it is relevant to demonstrate that the fluorescent label and the fluorescence method will not affect in anyway the selectivity of the aptamer probe for OPN. For that purpose, several experiments were carried out in which the aptamer solutions were added to several common extracellular proteins, such as BSA, thrombin and bovine OPN (an homologous of the human OPN) and fluorescence changes were recorded. These changes were then compared to the fluorescence change caused by human OPN.

As shown in Figure 14, it was found that the aptamer sensor yielded very weak fluorescence signals in response to other proteins such as BSA and thrombin, which contrasted with the pronounced fluorescence response to rhOPN. Moreover, the specificity of the aptamer to rhOPN could be further confirmed from the assays conducted with bovine OPN (homologous to the human OPN).

The results herein gathered revealed that the aptamer is selective for the human OPN, thus supporting its applicability for medical diagnosis. On the other hand, the fluorescence method was found to be easy, simple and accurate for the evaluation of the aptamer-protein complex formation. This procedure is straightforward and could be very simply used as an alternative to other existing techniques involving the use of aptamers as recognition elements (Z. Cao, 2002; Gokulrangan et al., 2005; Nutiu & Li, 2004; Stojanovic, Prada, & Landry, 2000; Zou et al., 2012; Zuo, Xiao, & Plaxco, 2009).

3.1.1.2. Development and optimization of ELISA`s method

To demonstrate the utility of the methodology implemented, the same method was applied to human thrombin, which plays an important role in the coagulation cascade and hemostasis. The thrombin binding aptamer (TBA) folds into a well-defined quadruplex structure and binds to its target with good specificity and affinity: TBA1FAM and TBA2FAM were used to perform the FA experiments. Interferences by other proteins were also evaluated.

In the experiments conducted with both TBA1FAM and TBA2FAM aptamers, the fluorescence profile revealed an increasing binding activity through fluorescence quenching as illustrated in Figures 15 and 16, respectively. These results indicate the specificity and affinity of the aptamers for both thrombin exosites. However, TBA1FAM displays higher fluorescence values and better stability after the saturation point has been reached. Furthermore, a linear response is reached at a concentration of 30.11 nM of human thrombin, which suggests that further studies should be conducted with this protein to evaluate possible differences in the binding affinity constants in shorter protein concentration intervals.

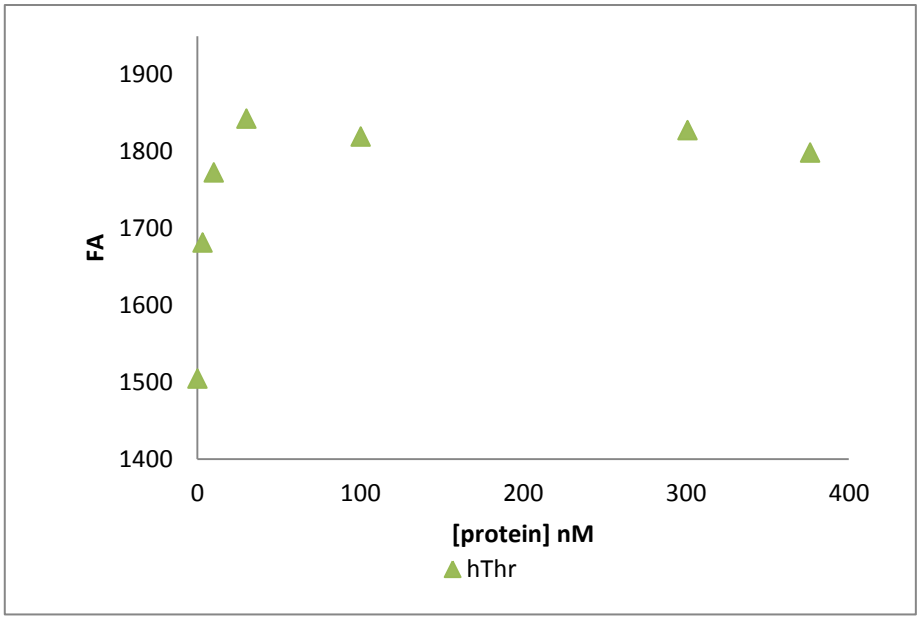

Figure 15 Fluorescence binding studies for TBA1FAM (20 nM) with concentrations from 0 to 376.31 nM of hThr, after 30 minutes incubation

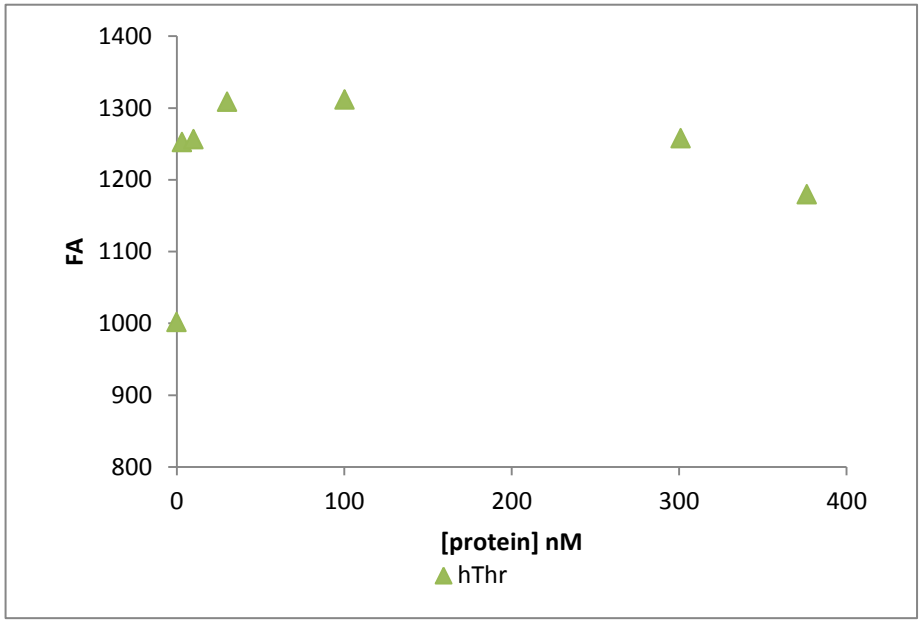

Figure 16 Fluorescence binding studies for TBA2FAM (20 nM) with concentrations from 0 to 376.31 nM of hThr, after 30 minutes incubation

The evaluation of possible unspecific binding of TBA1FAM and TBA2FAM was conducted using the same proteins that were previously used for the OPN-R3FAM aptamer. Similarly, these proteins were used as negative controls. Figures 17 and 18 illustrate the results obtained for TBA1FAM and TBA2FAM, respectively. In these experiments it was assumed that TBA is in a conformational equilibrium between its unfolded state and the folded, binding-competent G-

quartet conformation. Since thrombin binds only the G-quartet conformation, the target protein drives the equilibrium towards this state, thereby producing the observed signal. From the above mentioned figures, TBAFAM-aptamers do not bind specifically to BSA or to either of the human or bovine OPN evaluated. Furthermore, as OPN has a cleavage site to thrombin, it could have some binding affinity for thrombin DNA aptamers. Surprisingly, fluorescence values seemed more stable for BSA binding assays than for the OPN ones.

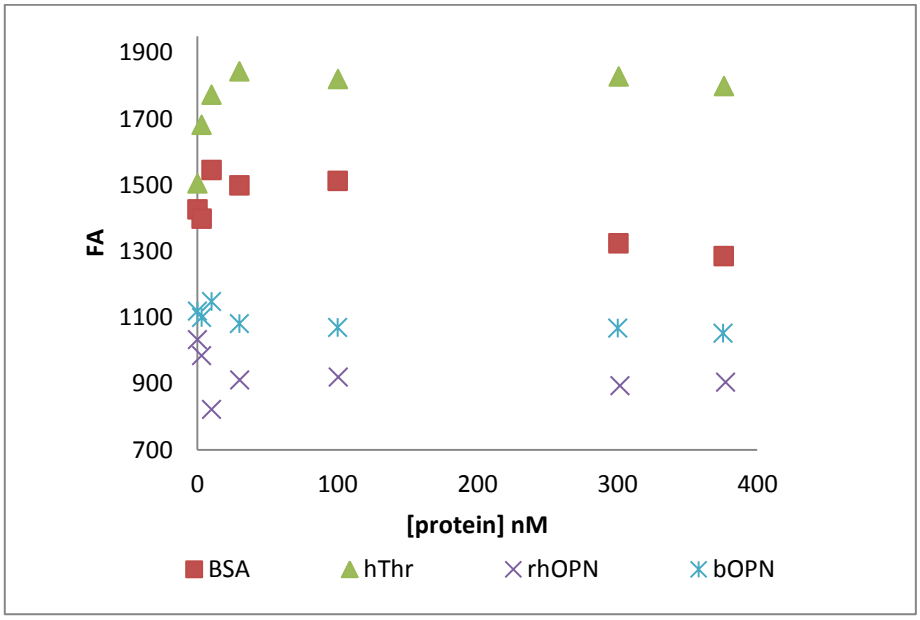

Figure 17 Plot 20nM TBA1FAM fluorescence responses at 528nm versus protein concentrations (bovine osteopontin, bOPN, bovine serum albumin, BSA, human blood thrombin, Thr and human osteopontin, rhOPN) in the standard phosphate binding buffer, after 30 minutes incubation

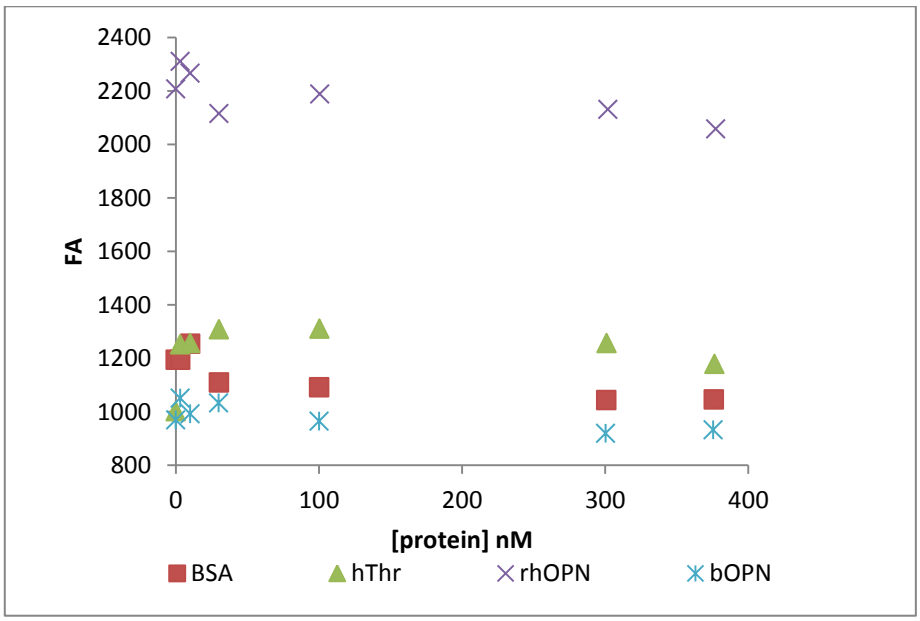

Figure 18 Plot 20nM TBA2FAM fluorescence responses at 528nm versus protein concentrations (bovine osteopontin, bOPN, bovine serum albumin, BSA, human blood thrombin, Thr and human osteopontin, rhOPN) in the standard phosphate binding buffer, after 30 minutes incubation

Again, the results herein obtained revealed a good selectivity of the TBA aptamers which turns ELISA into a valuable method to assess the specificity of aptamers for their target proteins so that they could be used in electrodes to develop detection aptasensors. Despite its long use for many biotechnological applications, ELISA is currently being used as a pioneering concept (Chang, Tang, Wang, Jiang, & Li, 2010; H. Chen et al., 2013; Jin, Bai, & Li, 2010; Paborsky et al., 1993; W. Wang, Chen, Qian, & Zhao, 2008).

3.1.1.3. Dissociation constant (K_d)

The analysis of affinity assays conducted with thrombin using a wide range of measured concentrations could be achieved with TBA affinity probes. These TBA aptamers bind to thrombin with significantly different equilibrium constants (K_d). The dissociation constant (K_d) is a parameter that reflects the amount of complex formed for a specific recognition element, i.e. for each TBA, using a specific detection method. In addition, the smaller the K_d, the stronger the link between the aptamer and the protein will be.

Initial non-linear fitting analysis of the fluorescence data obtained with the two aptamers (as described in the Materials and Methods section) demonstrated that the model 1:1 was adequate to fit the results (Table 7).

Table 7 Dissociation constants (K_d) for TBA1 and TBA2 with different protein targets for two different incubation times

K_d value (nM)	Incubation time	hThr	rhOPN	bOPN	BSA
TBA1FAM	30 min	1.820	*	*	*
	4 h	1.071	*	*	*
TBA2FAM	30 min	0.867	DNC	DNC	*
	4 h	1.778	DNC	*	DNC

DNC – does not converge, *it makes no sense to calculate the K_d value for proteins for which the aptamer was not specific. Therefore, the calculated values are merely obtained by fitting approximations. Designations: bovine Osteopontin (bOPN), bovine serum albumin (BSA), human blood thrombin, (hThr) and human Osteopontin (rhOPN).

The non-linear fitting of the data enabled the estimation of the K_d values for each TBA aptamer, when the TBA-thrombin complex was formed.

TBA1 was the first DNA thrombin binding aptamer to be selected and its dissociation constant was previously reported to be around 26 nM (Bock, 1992). Furthermore, this aptamer was

described as a potential anticoagulant agent, and presents enzymatic inhibitory functions that are required for thrombin-mediated coagulation since it interacts with the fibrinogen-binding exosite-1 of thrombin (Chiu & Huang, 2009). Most studies conducted with TBA1 aptamer reported K_d values around 25 nM (Hamula, Guthrie, Zhang, Li, & Le, 2006). However, in the current study (Table 8), a K_d value of 1.820 nM was reached. This result suggests that the TBA1FAM has a better specificity for thrombin than the TBA1 aptamers used in the other reported studies. Comparing K_d values for TBA1 aptamer using fluorescence anisotropy (Table 2) with the ones described by Hamaguchi and collaborators (2001) it can be concluded that the fluorescence measurement was more sensitive than their approach. In their research, using an aptamer-beacon and the fluorescence as the detection technique, they obtained a K_d value of 10 nM.

Regarding the TBA2, it was first described by Tasset *et al.* (1997) that reported its high affinity to thrombin, K_d = 0.5 nM. As can be seen in Table 7, the value estimated for this aptamer in the current work is close to the K_d value previously reported, suggesting that TBA2 behaves in a more predictable and constant manner, and also that the fluorescence method is as sensitive as the methods used in other studies (Tasset et al., 1997).

An attempt to fit the data obtained in the assays conducted with interfering proteins was done. However, since the thrombin aptamers are non-specific to those proteins, no protein-aptamer complexes were formed, and therefore no consistent changes could be observed in the fluorescence, as expected.

3.1.1.4. EMSA as an analytical technique for detection of the formation of the aptamer-protein complex by EMSA

3.1.1.4.1. Validation of the TBA1FAM and TBA2FAM aptamers binding to thrombin by EMSA

Before conducting the EMSA binding studies, several optimization steps were performed to guarantee the best gel resolution and detection limit of the samples. These optimization steps consisted in evaluating several operational parameters that include the gel running temperature (4°C and room temperature); the presence or absence of stacking gel; and different TBE concentrations used in the gel (TBE 0,5X or TBE 5X).

The best conditions for EMSA binding assays were determined as: presence of a 4% stacking gel; use of TBE 5X for gel preparation; and gel running at room temperature. The EMSA studies were conducted with the FAM labeled aptamers, namely TBA1FAM and TBA2FAM.

Calibration curves were conducted for both aptamers and a significant aptamer concentration of 0,8 μM was necessary to enable the samples detection (Figure 19).

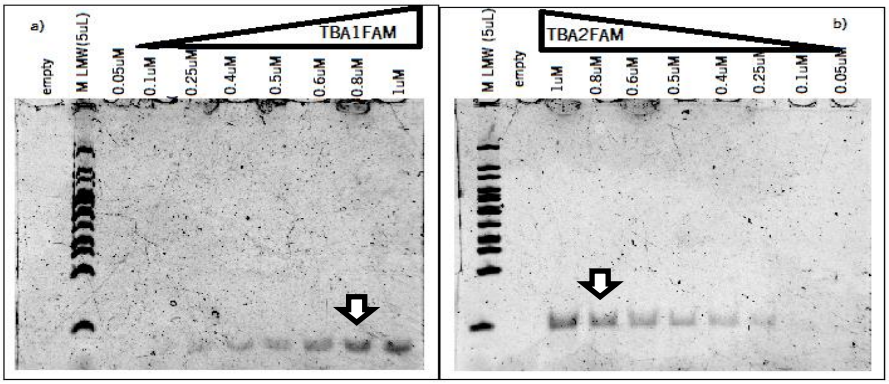

Figure 19 Electrophoretic Mobility Shift Assay (EMSA) using different concentrations of a) TBA1FAM and b) TBA2FAM under the conditions described in Materials and Methods section. Binding reaction mixtures were applied on a 12 % non-denaturing gel containing 5X TB TBE buffer. The mobility of free aptamers, stained by SybrGreen Safe®, was detected using the Chemidoc System.

3.1.1.4.2. Optimization of the method to TBA1FAM and TBA2FAM aptamers binding to thrombin by EMSA

After measuring fluorescence in the ELISA, 10 μL of each binding reaction mixture was used to perform the EMSA analysis. In these experiments, it was expected that starting from lower thrombin concentrations the band correspondent to the free aptamer would gradually disappear, and at the same time the band with lower mobility corresponding to TBA-thrombin complex would appear (Figure 20). However, for unknown reasons, the presence of the aptamer-protein complex (low mobility band) could not be detected for both thrombin aptamers. This could be due to the fact of being operating in conditions below the limits of detection of the method. In addition, using the samples from ELISA assays to prepare the EMSA experiment will dilute the samples even more. Sosic *et al.* (2010) found that the minimum protein concentration required to form the aptamer:protein complex was about 10 μM. In the current work, protein concentrations lower than this reference value was used. Following this assumption it is expected that there is no sufficient amount of protein in order to see it in the gel.

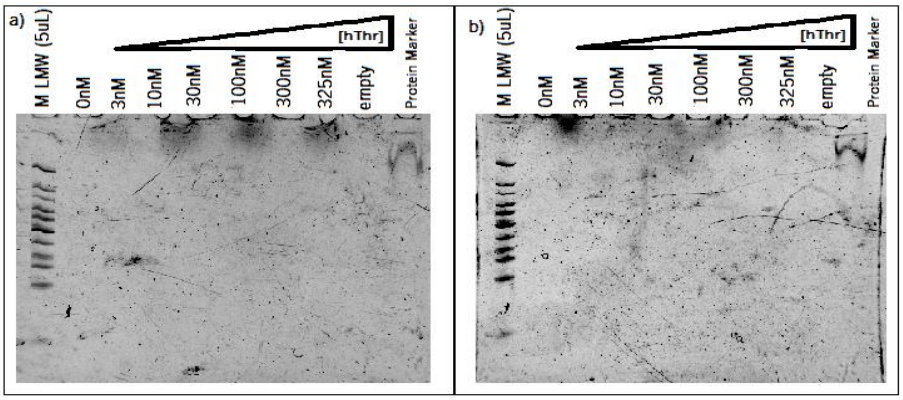

Figure 20 Electrophoretic Mobility Shift Assay (EMSA) of a) TBA1FAM and b) TBA2FAM under increasing concentrations of human thrombin, collected from ELISA assays. Binding reaction mixtures were applied on a 12% non-denaturing gel containing 5X TBE buffer. The mobility of free aptamers, stained by SybrGreen Safe®, was detected using the Chemidoc System.

Figure 21 shows the conducted assays with TBA1FAM and TBA2FAM with interfering proteins (BSA, rhOPN and bOPN) demonstrated that no binding occurred between these proteins and the aptamers, which is in accordance with the previous fluorescence results. Nevertheless, the overall results gathered in the EMSA assays were inconclusive since no protein-aptamer complexes could be observed in the experiments in which human thrombin was used. Furthermore, it is important to mention that the gels were also stained using the silver nitrate method. However, it was still not possible to observe any aptamer-protein complex in the gels, which may be the result of the low protein concentrations kept concerning the dilutions made for EMSA proceedings.

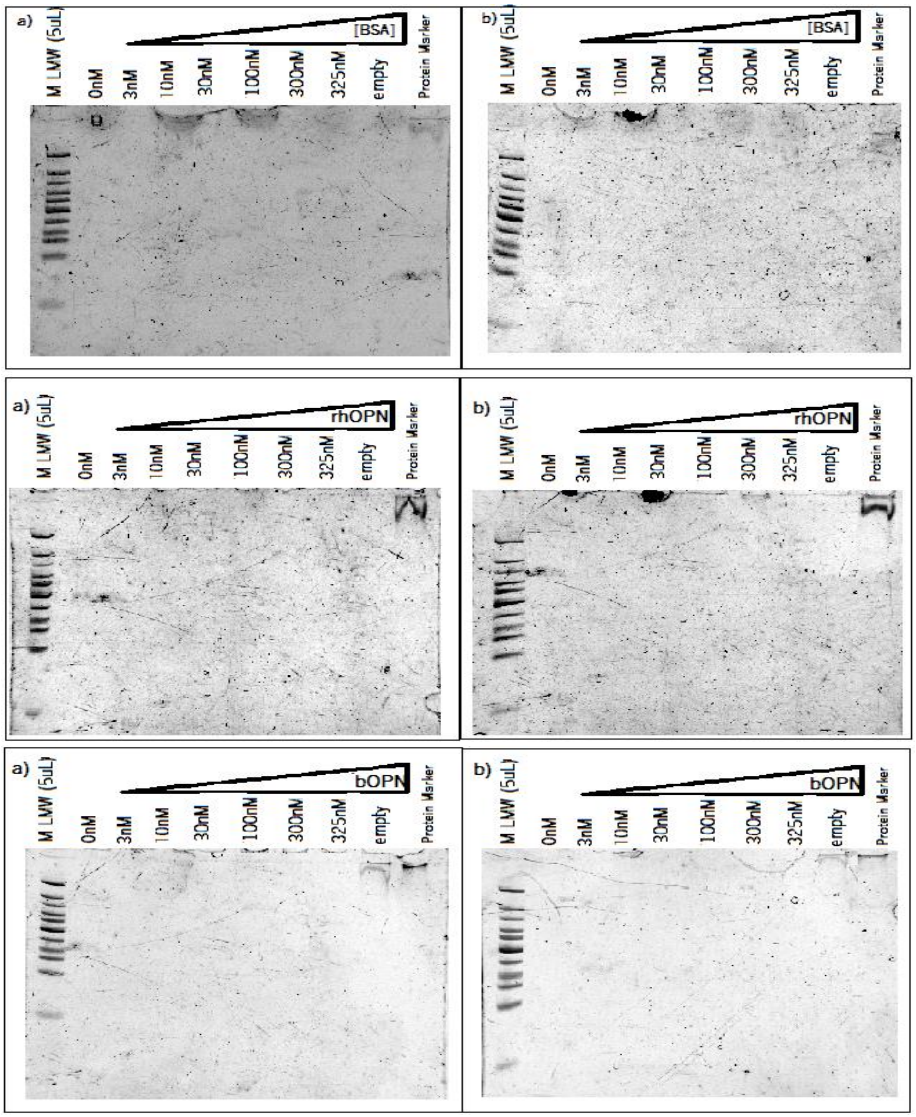

Figure 21 Electrophoretic Mobility Shift Assay (EMSA) of a) TBA1FAM and b) TBA2FAM under increasingly concentrations of interferents, harnessed from ELISA assays. Binding reactions were applied on a 12% non-denaturing gel containing 5X TBE buffer. The mobility of free aptamers, stained by SybrGreen Safe®, was detected using the Chemidoc System.

3.2. Development, optimization and validation of an aptasensor for thrombin

The progress of the Human Genome Project has generated substantial interest in the use of nucleic acid technologies to develop sensors capable of identifying human proteins involved in crucial biological processes. The challenges for biomedical researchers include the characterization of the biological function of these proteins. Intense research activities are being carried out worldwide to apply this knowledge for the design and engineering of specific, fast, simple, and

sensitive electrochemical aptasensors for medical diagnostics applications (Hong et al., 2012; Mir et al., 2006).

3.2.1. Optimization of experimental conditions for aptasensor

Several parameters were further considered to optimize the aptasensor regarding its sensitivity and accuracy including the TBA-thrombin incubation and the sensor operating conditions.

3.2.1.1. Optimization of the sensor conditions

The evaluation of different scan rates is presented in Figure 22. The electrode was incubated with 60 µl of the redox solution and 4 different scan rates were tested, namely 0,01 V/s; 0,05 V/s; 0,1 V/s and 1 V/s. After analyzing the data, the best scan rates, i.e. the ones that presented a lower inconsistency between 5 consecutive scans were 0,01 V/s and 0,05 V/s. In the present work the scan rate of 0,05 V/s was chosen since it presented a good compromise between scan reproducibility and analysis speed.

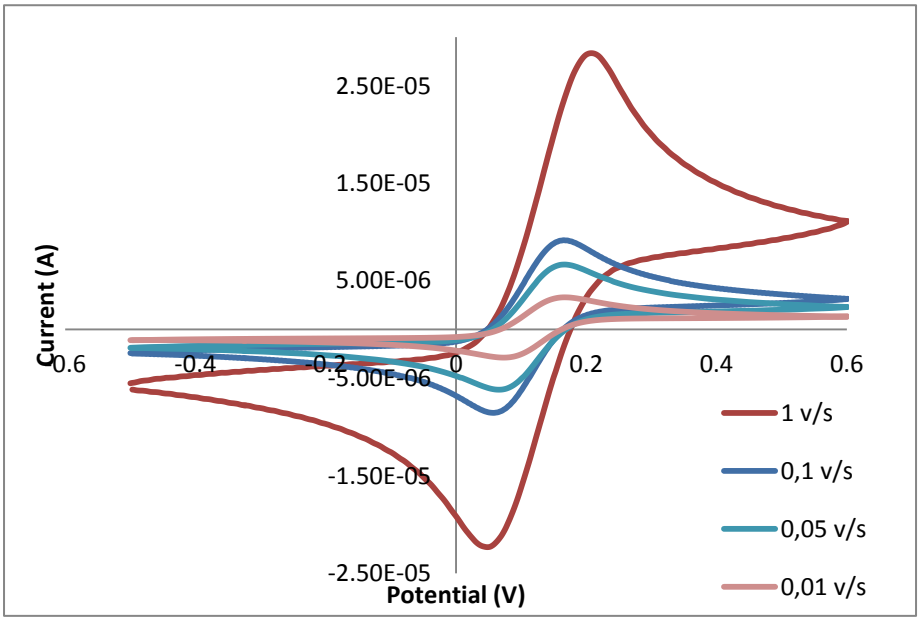

Figure 22 Cyclic voltammetry scan rate optimization results (0,01; 0,05; 0,1 and 1 v/s) using a $[Fe(CN)_6]^{-3/-4}$ redox probe

In order to avoid any interference related with the preparation of the redox solution, a routine preparation protocol was developed. In fact, when working with aptamers the addition of high concentrations of salt (often 2 M NaCl or KCl) can cause the disruption of the hydrogen bonds and electrostatic interactions that are responsible for most of the aptamer-target association. In addition, it is well-known that for the optimal performance of electrochemical detection methods, the presence of counter-ions in the medium is desirable. However, it has been demonstrated that

the presence of certain ions can induce the folding of some aptamers in a manner similar to their molecular targets, as is in fact the case with TBA in the presence of KCl (A. E. Radi et al., 2006). Therefore, following a daily protocol in which the concentration of each reagent is controlled minimizes possible interference in the signals obtained by CV.

3.2.1.2. Optimization of the incubation conditions

The incubation time of thrombin with the aptamer was found to have a major impact on the formation of the aptamer-target complex. Several studies showed that a time range between 30 and 40 min is enough to ensure quenching between the aptamer and the particular protein (Ji et al., 2012; Khezrian, Salimi, Teymourian, & Hallaj, 2013; S. Xie et al., 2012). Therefore, 30 min incubation time was selected to perform binding assays in the current work.

Thrombin-aptamers prefer to adopt a G-quarter structure when binding to thrombin (Ho & Leclerc, 2004). Incubation conditions, such as the pH value and temperature have a great impact on the formation of the G-quarter structure (Suprun et al., 2008). Some authors showed that the protonation of guanine groups of the aptamer sequence occurs at pH values below 7.0, while an increasing in the aptamer negative charge was seen for pH values above 8.0, thus leading to the disassemble of the G-quartets of aptamer (Zhao et al., 2011) Based on this, a redox solution containing 5 mM $[Fe(CN)_6]^{4-/3-}$ in 100 mM KCl prepared in PBS, pH 7.4 was selected for the SPE preparation and detection of hThr in the current study.

The effect of the incubation temperature is also a crucial point to consider. Tang and Shafer (2006) suggested that thrombin does not bind the aptamer when the incubation temperature approaches or surpasses its melting temperature (46◦C). Other authors reported that the highest signal could be obtained with an incubation temperature of 4◦C, in which the G-quarter structure was stable (Eva Baldrich, Restrepo, & O′ Sullivan, 2004). However, in the current study the immobilization assays were conducted at RT since the fluorescence binding assays were previously performed in those conditions.

3.2.2. Design of an electrochemical aptasensor

TBA1 (15-mer) or TBA2 (26-mer) aptamers were modified with biotin at 5`- end as the following sequences: (TBA1-biotin): 5'-biotin-GGT TGG TGT GGT TGG-3' and (TBA2-biotin): 5'-biotin-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3'. These aptamers are then attached individually in

different SPE`s via biotin- avidin binding. This bioaffinity method facilitates the immobilization of TBA on a SPE without losing its binding properties.

In a study conducted by Diculescu et al., (2010), it has been shown that in the presence of alkali metals, such as potassium ions presented in the phosphate buffer solution, TBA forms an antiparallel intramolecular quadruplex consisting of two G-quartets connected by two TT loops and one TGT loop. The formation of the quadruplex structure plays an important role in binding to thrombin. Besides, due to its low cost and good redox characteristics, potassium ions were used as electrochemical indicator in our proposed system.

A redox couple solution composed of 5 mM $[Fe(CN)_6]^{4-/3-}$ in 100 mM KCl was used to characterize the electrochemical behavior of the bare sensor by cyclic voltammetry (CV). It could be observed the binding of a quasi-reversible and well-defined redox peak from $[Fe(CN)_6]^{4-/3-}$ (dark blue voltammogram). After the electrode modification with DSP, avidin and BSA the current responses of the SPE were significantly lower than that of the bare electrode, which suggested that these modifications could greatly promote the electron transfer. After the immobilization of TBA-biotin, the response was clearly reduced due to the electrostatic repulsion between $[Fe(CN)_6]^{4-/3-}$ and the negatively charged aptamer (orange curve). The resulting voltammograms are illustrated in Figure 23.

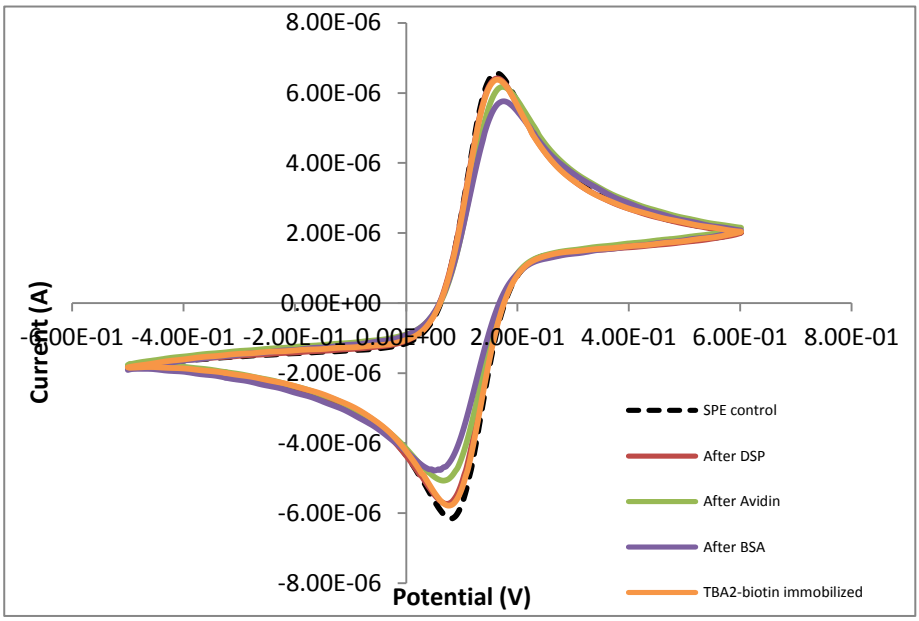

Figure 23 Cyclic voltammograms for a thrombin TBA2 aptasensor regarding the $[Fe(CN)_6]^{-3/-4}$ redox probe: SPE control (voltammogram to the electrode itself), After DSP (voltammogram after DSP modification), After Avidin (voltammogram after Avidin incubation, After BSA (voltammogram after BSA blockage), TBA2-biotin immobilized (voltammogram after aptamer immobilization)

3.2.3. Electrochemical detection of thrombin using the aptasensors TBA1-biotin and TBA2-biotin

CV analysis were conducted against a series of hThr concentrations ranging from 0.025 nM to 50 nM using an SPE in which 1 μM TBA1-biotin aptamer was immobilized. The concentration-dependent decrease of the current was observed (Figure 24).

From Figure 24 it could be seen that TBA1-biotin was not able to describe the current variations as a function of the thrombin concentration. Further experiments were conducted in order to overcome this issue and improve the thrombin detection.

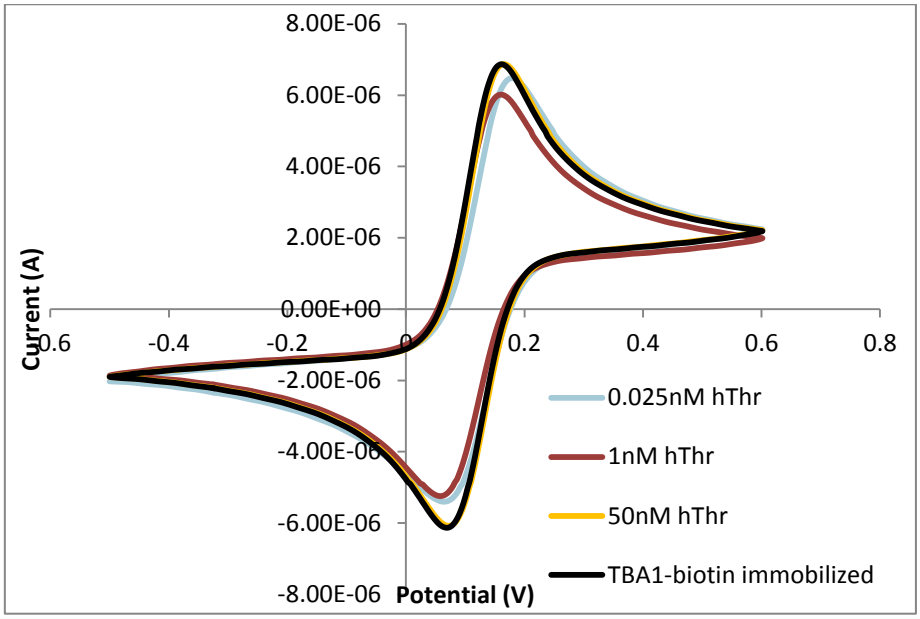

Figure 24 Electrochemical analysis of human thrombin hThr using modified TBA1-biotin-aptamer - immobilized on screen-printed electrode chip: a considerable current drop occurred by the treatment of hThr in a range of 0.025 nM–50nM from cyclic voltammetry; scan rate: 0.05V/s with a step potential of -0.5V to -0.6V/s, using 5mM $[Fe(CN)_6]^{4/3-}$ in 100mM KCl prepared in PBS, pH 7.4

For that purpose, the concentration of aptamer used for immobilization was reduced from 1μM to 0.5 μM of TBA1-biotin, and only two different hThr concentrations were tested, as illustrated in Figure 25.

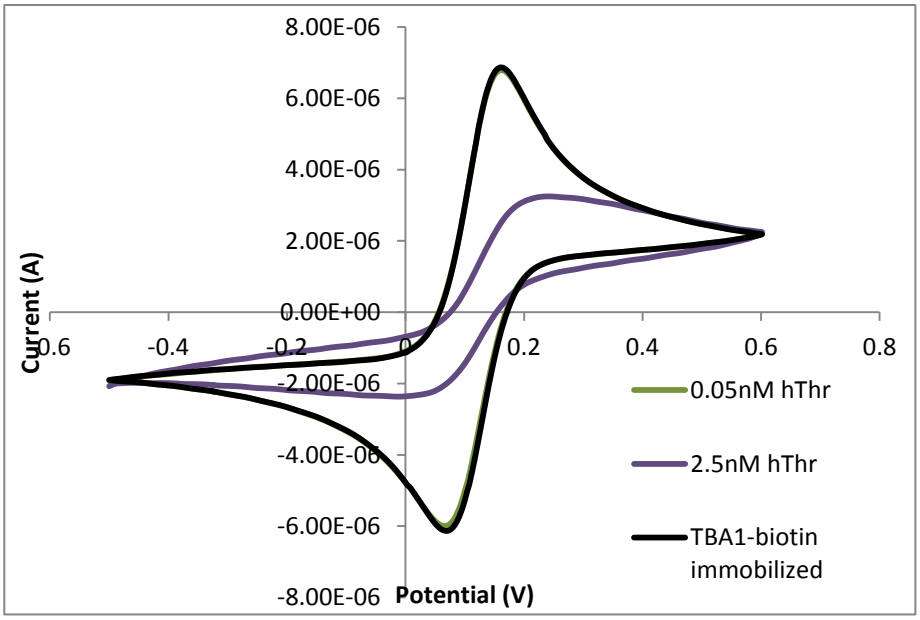

Figure 25 Electrochemical analysis of human thrombin (hThr) using modified (0.5 μM) TBA1-biotin-aptamer - immobilized on screen-printed electrode chip: a considerable current drop was occurred by the treatment of hThr in a range of 0.05 nM and 50nM from cyclic voltammetry; scan rate: 0.05V/s with a step potential of -0.5V to -0.6V/s, using 5mM $[Fe(CN)_6]^{4/3-}$ in 100mM KCl prepared in PBS, pH 7.4

However, the current drastically decreased with a thrombin concentration of 0.025 nM hThr and this was taken as a background signal. The results suggest that the aptamer gets saturated at very low thrombin concentrations and this precludes the use of TBA1-biotin aptamer in the next recognition steps. Neither is necessary to note that the concentration of 50 nM aptamer is already saturated and out of the detection range.

On the other hand, TBA2-biotin exhibits a reduction peak increasingly smaller upon thrombin addition from a concentration above 50 nM even if the linearity was reached between 0.5-50 nM of thrombin concentration (Figure 26). For this aptamer the current change as a result of the thrombin concentration was found to be saturated at 0.05 nM of hThr.

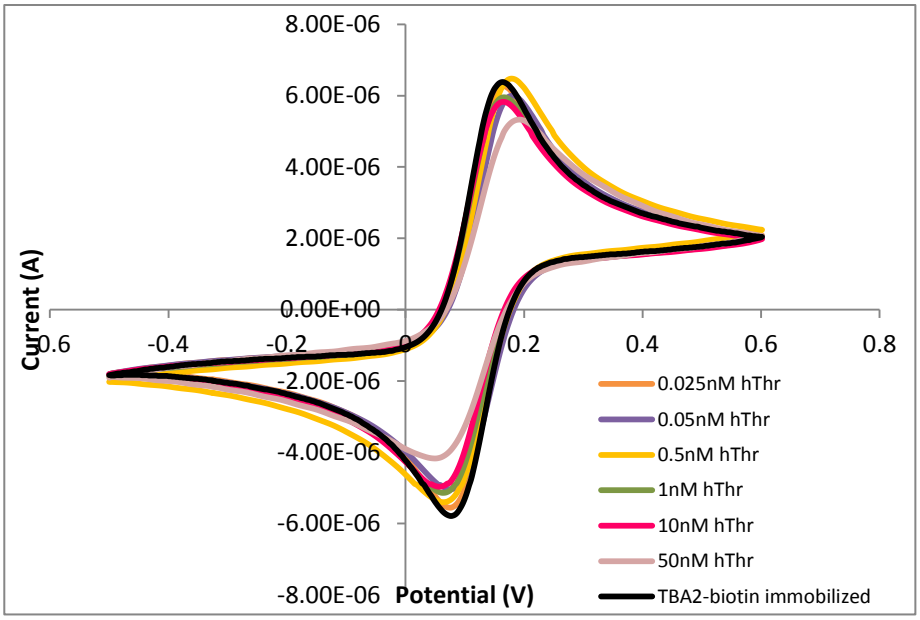

Figure 26 Electrochemical analysis of human thrombin (hThr) using modified TBA2-biotin-aptamer - immobilized on screen-printed electrode chip: a considerable current drop was occurred by the treatment of hThr in a range of 0.025 nM–50nM from cyclic voltammetry; scan rate: 0.05V/s with a step potential of -0.5V to -0.6V/s, using 5mM $[Fe(CN)_6]^{4/3-}$ in 100mM KCl prepared in PBS, pH 7.4

Binding Thr to the aptamer immobilized on the SPE surface may have affected the rate of electron flow produced from the redox reaction between the two reagent of the redox probe ($[Fe(CN)_6]^{4/3-}$). Therefore, the decrease in current after hThr incubation was associated to the specific interaction between aptamer and hThr that influenced the current. Studies on the interaction between aptamers and small molecules indicated that low-molecular targets are often pocketed into the folded aptamer structure, and it is difficult to perform a sandwich assay. A simple single-site binding assay is generally limited for the development of a sensitive EC aptasensor for small molecules since the electron transfer resistance or current changes due to the small target binding is usually low when compared to macromolecules like proteins. Binding thrombin to its aptamer leads to a decrease in the current due to interference with the electron flow. Therefore, in this study, it is believed that hThr induced the folding of the aptamer through the formation of a stem-loop structure, which probably induced the observed changes in the electrostatic property (Xiao et al., 2005).

3.2.4. Determination of the SPE linear response range for the detection of thrombin

The analytical performance of the aptasensor for thrombin was assessed under the optimal experimental conditions above mentioned. As shown in Figure 26, the electrocatalytic currents decreased with the increase of thrombin concentrations. Plotting the current response versus the thrombin concentration (Figure 26), it was possible to realize that the sensor was answering accordingly to thrombin concentrations. However, a linear response range for the detection of thrombin, it was only obtained for thrombin concentrations ranging from 0.5 to 50 nM, as shown in Figure 27.

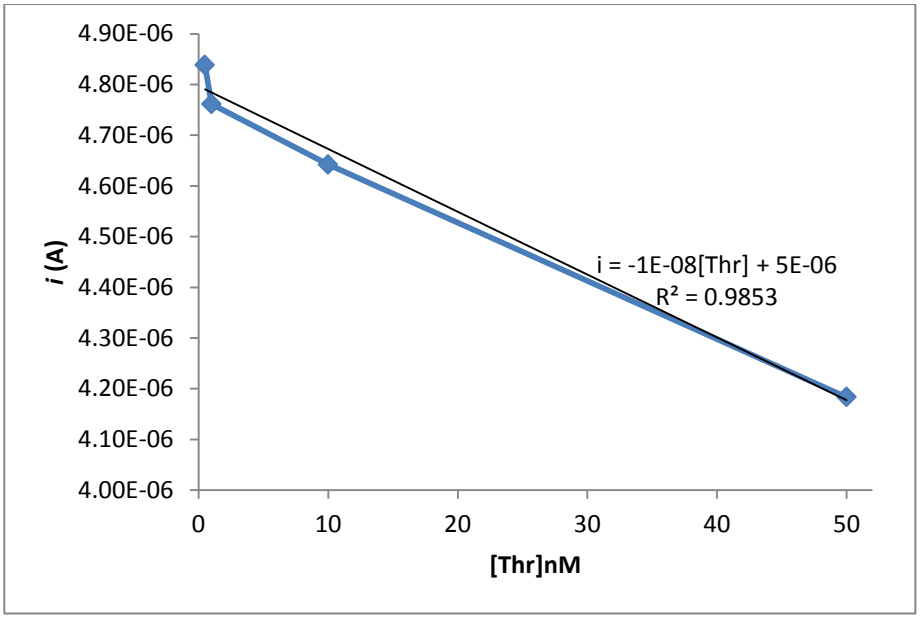

Figure 27 Thrombin concentration versus oxidation peak. The concentration of the aptamer employed during sensor fabrication was 1 μM. The incubation time was 30 minutes at RT

An equation that describes the linear response was defined as follows: i(A)= $1e^{-8}$ [hThr] + $5e^{-6}$ where i is the current response in relation to the oxidation peak and [hThr] is the thrombin concentration. The correlation coefficient is 0.9853 which indicated a good fitting of the experimental data by linear regression. Furthermore, it was found that the low detection limit was 0.025 nM when in (Frense et al., 2013) it was referred that thrombin concentration in blood is normally around 0.01 nM.

The results herein gathered showed that the thrombin aptasensor for thrombin detection presents a higher sensitivity, but lower detection limit, as compared with other reported methods in Table 4. In addition, after technical refining, it is believed that this aptasensor could be used for further thrombin detections to assess aptasensor performance.

3.2.4.1. The selectivity of the aptasensor

The specificity performance of the aptamer-based assay for thrombin was further studied in this work. For that purposed, three interfering proteins were tested instead of thrombin using the same experimental procedure. These interfering proteins are either abundant proteins in serum (e.g., BSA) or analogous to the thrombin such as bovine Osteopontin (bOPN) and human Osteopontin (hOPN). Comparison of the CV responses of the biosensor were performed when exposed to hThr (50 nM), hOPN (50 nM), BSA (50 nM) and bOPN (50 nM), though, the regeneration step was not accomplished, considerations about the selectivity of the aptasensor cannot be done.

3.2.4.1. Sensor regeneration and reusability

Similarly to other aptasensors described in the literature, the thrombin aptasensor herein developed should be prone to regeneration and reusable. Several known regeneration reagents would be evaluated including 6 M guanidine hydrochloride (Xiao et al., 2005), 7 M urea buffer (Y. Liu et al., 2010), 2 M NaCl, 10% SDS and 1 M HCl (A. E. Radi et al., 2006).

However, the regeneration step was only tested with 7 M urea buffer for 2 minutes.The regeneration steps for the removal of hThr from the TBA2-aptasensor were not effective. No recovery could be observed given the oxidation peak after hThr detection. Studies developed by Radi and coworkers used a molecular beacon aptasensor by simply unfolding the aptamer in 1.0 M HCl. The aptasensor could be regenerated for 25 times no loss in electrochemical signal upon subsequent thrombin binding (A. E. Radi et al., 2006).

To sum up, given the importance of hThr as a clinical marker in the diagnosis of pulmonary metastasis and when searching for anti-coagulants and antithrombotic agents to interfere with blood coagulation, the ability to selectively detect hThr molecules in the presence of overabundant non-specific proteins augurs well for future uses of this aptasensor in clinical diagnostics (Chiu & Huang, 2009). Compared to antibody-based affinity biosensors, aptasensors provide the advantage of being chemically stable. The chemical stability of nucleic acids ensures that aptasensors may be regenerated under conditions that disrupt aptamer-protein complex; however with TBA2-biotin this regeneration buffer does not remain advantageous.

4. Conclusion

A new strategy to convert efficiently the aptamer-target recognition event into a measurable signal was described in the present work by introducing an fluorescent label reporter into the standard thrombin aptamer sequences. The extended aptamers seem to maintain the binding properties of the original sequences and preserve its high affinity and selectivity. An imaging system was used to measure fluorescence, thus enabling the determination of anisotropy changes in multiple samples. The fluorescence detection methods allows rapid and efficient formation of the aptamer-protein complex allowing this bond measure and determine the dissociation constants that are one important feature of aptamers which were taken into consideration for the construction of the aptamer. Fluorescence anisotropy technique has the unique ability of effectively detecting protein interactions with other molecules.

The idea of coupling a fluorophore to the aptamer enabled the detection of the aptamer-protein complex formation and the further determination of the binding dissociation constants. Dissociation constants for TBA1FAM and TBA2FAM were estimated as 1.820 nM and 0.867 nM, respectively. The fluorescence binding experiments herein conducted led to K_d values for these aptamers that are in accordance with the values reported in the literature, being in some cases even lower which indicates the sensitivity of the analytical method.

The method used to develop the sensor was efficient because all samples dropped in the electrode showed a profile in which each concentration described a hThr voltamogramm. Thus, an aptamer-based electrochemical biosensor was developed for the detection of thrombin. Biotin modified TBA1 and TBA2 aptamers were immobilized on the surface of a screen-printed electrode, and the electrochemical analysis was conducted by cyclic voltammetry using $[Fe(CN)_6]^{4/3-}$ solution as a redox probe. The TBA2 electrochemical aptasensor was found to be sensitive to hThr with a linear response range from 0.5 to 50 nM and a LOD of 0.025 nM.

TBA2 aptasensor could be viewed as an emerging device in the biomedical field and shows significant promise in the detection, analysis and monitoring of thrombin. However, there are some issues that need to be overcome. No effect was observed of interfering proteins in the response of the electrode, because the non-efficiency regeneration protocol. There are some issues to be improved such as the sensitivity, the sensor regeneration and the interferences which could lead to further research.

5. Future work: Improving and refining the techniques

The fluorescence anisotropy technique used in this work can be further improved. An alternative dye can be used in these experiments, as for example the Cy5 dye. It has much longer excitation and emission wavelengths than 6-FAM. Longer excitation wavelength will cause less photobleaching and reduce the issues associated with the samples autofluorescence (Z. Cao, 2002).

Furthermore, regarding the aptasensor performance, some parameters should be reviewed to improve the sensor's sensitivity and selectivity for thrombin detection. In particular, the parameters associated with the incubation of the protein in the electrode could be re-evaluated. As previously mentioned, some authors reported that the incubation temperature should not approach or surpass thrombin melting temperature since the aptamer cannot maintain its folded structure (Tang & Shafer, 2006), while other authors determined that low temperatures lead to more stable G-quarter structures of thrombin-aptamer, thus better detection (Eva Baldrich et al., 2004; Zhao et al., 2011) .Therefore, a low incubation temperature (4ºC) could be tested in the development of the thrombin aptasensor towards an improvement of its sensitivity.

Finally, the advent of biosensors has allowed biomedical research and clinical diagnostics to develop upon the advantages of miniaturization, such as reduced sample volumes, faster reaction times, and the possibility of multiplexed detection (Ng & Chong, 2011). Multiplexing, meaning try to immobilize more than one specific aptamer in electrodes it is of particular importance, since the simultaneous detection of multiple targets at once can result in significant time savings, particularly for applications requiring high-throughput. Often, multiple targets must be detected in order to draw a meaningful conclusion in clinical diagnosis. Therefore, immobilizing both TBA1 and TBA2 aptamers in a single SPE could be an interesting and brainy approach to develop.

6. References

Abe, K., Yoshida, W., & Ikebukuro, K. (2013). Electrochemical Biosensors Using Aptamers for Theranostics. *Adv Biochem Eng Biotechnol*.

Alonso-Lomillo, M. A., Dominguez-Renedo, O., Matos, P., & Arcos-Martinez, M. J. (2009). Electrochemical determination of levetiracetam by screen-printed based biosensors. *Bioelectrochemistry, 74*(2), 306-309.

Bai, J., Wei, H., Li, B., Song, L., Fang, L., Lv, Z., Wang, E. (2008). [Ru(bpy)2(dcbpy)NHS] labeling/aptamer-based biosensor for the detection of lysozyme by increasing sensitivity with gold nanoparticle amplification. *Chem Asian J, 3*(11), 1935-1941.

Balamurugan, S., Obubuafo, A., Soper, S. a., & Spivak, D. a. (2008). Surface immobilization methods for aptamer diagnostic applications. *Analytical and bioanalytical chemistry, 390*, 1009-1021.

Baldrich, E., Restrepo, A., & O'Sullivan, C. K. (2004). Aptasensor development: elucidation of critical parameters for optimal aptamer performance. *Anal Chem, 76*(23), 7053-7063.

Baldrich, E., Restrepo, A., & O'Sullivan, C. K. (2004). Aptasensor Development: Elucidation of Critical Parameters for Optimal Aptamer Performance. *Clin Chem, 45*, 1628-1650.

Bang, G. S., Cho, S., & Kim, B. G. (2005). A novel electrochemical detection method for aptamer biosensors. *Biosens Bioelectron, 21*(6), 863-870.

Beausoleil, M. S., Schulze, E. B., Goodale, D., Postenka, C. O., & Allan, A. L. (2011). Deletion of the thrombin cleavage domain of osteopontin mediates breast cancer cell adhesion, proteolytic activity, tumorgenicity, and metastasis. *BMC Cancer, 11*(1), 25.

Bock, P. E. (1992). Active-site-selective labeling of blood coagulation proteinases with fluorescence probes by the use of thioester peptide chloromethyl ketones. II. Properties of thrombin derivatives as reporters of prothrombin fragment 2 binding and specificity of the labeling approach for other proteinases. *J Biol Chem, 267*(21), 14974-14981.

Bohunicky, B., & Mousa, S. A. (2010). Biosensors: the new wave in cancer diagnosis. *Nanotechnology, Science and Applications*, 1.

Bunka, D. H., & Stockley, P. G. (2006). Aptamers come of age - at last. *Nat Rev Microbiol, 4*(8), 588-596.

Burmeister, P. E., Lewis, S. D., Silva, R. F., Preiss, J. R., Horwitz, L. R., Pendergrast, P. S., Keefe, A. D. (2005). Direct in vitro selection of a 2'-O-methyl aptamer to VEGF. *Chem Biol, 12*(1), 25-33.

Cai, H., Lee, T. M.-H., & Hsing, I. M. (2006). Label-free protein recognition using an aptamer-based impedance measurement assay. *Sensors and Actuators B: Chemical, 114*(1), 433-437.

Cao, D. X., Li, Z. J., Jiang, X. O., Lum, Y. L., Khin, E., Lee, N. P., Luk, J. M. (2012). Osteopontin as potential biomarker and therapeutic target in gastric and liver cancers. *World J Gastroenterol, 18*(30), 3923-3930.

Cao, Z. (2002). *Using Fluorescence Anisotropy for Sensitive Platelet-Derived Growth Factor Detection based on Molecular Aptamers.* Master, Florida, University of Florida.

Centi, S., Tombelli, S., Minunni, M., & Mascini, M. (2007). Aptamer-based detection of plasma proteins by an electrochemical assay coupled to magnetic beads. *Anal Chem, 79*, 1466-1473.

Chang, H., Tang, L., Wang, Y., Jiang, J., & Li, J. (2010). Graphene Fluorescence Resonance Energy Transfer Aptasensor for the Thrombin Detection. *Anal Chem, 82*, 2341-2346.

Chen, H., Yuan, F., Wang, S., Xu, J., Zhang, Y., & Wang, L. (2013). Aptamer-based sensing for thrombin in red region via fluorescence resonant energy transfer between NaYF4:Yb,Er upconversion nanoparticles and gold nanorods. *Biosens Bioelectron, 48*, 19-25.

Chen, L. J., Seo, J. H., Eller, M. J., Verkhoturov, S. V., Shah, S. S., Revzin, A., & Schweikert, E. A. (2011). Quantitative label-free characterization of avidin-biotin assemblies on silanized glass. *Anal Chem, 83*(18), 7173-7178.

Chiu, T. C., & Huang, C. C. (2009). Aptamer-functionalized nano-biosensors. *Sensors (Basel), 9*(12), 10356-10388.

Cho, E. J., Collett, J. R., Szafranska, A. E., & Ellington, A. D. (2006). Optimization of aptamer microarray technology for multiple protein targets. *Anal Chim Acta, 564*(1), 82-90.

Cho, E. J., Lee, J. W., & Ellington, A. D. (2009). Applications of aptamers as sensors. *Annu Rev Anal Chem (Palo Alto Calif), 2*, 241-264.

D'Orazio, P. (2003). Biosensors in clinical chemistry. *Clinica Chimica Acta, 334*(1-2), 41-69.

Daniels, J. S., & Pourmanda, N. (2007). Label-Free Impedance Biosensors Opportunities and Challenges. *Electroanalysis, 19*(12), 1239-1257.

Diculescu, V. C., Chiorcea-Paquim, A. M., Eritja, R., & Oliveira-Brett, A. M. (2010). Thrombin-Binding Aptamer Quadruplex Formation: AFM and Voltammetric Characterization. *J Nucleic Acids, 2010*.

Ding, C., Ge, Y., & Lin, J. M. (2010). Aptamer based electrochemical assay for the determination of thrombin by using the amplification of the nanoparticles. *Biosens Bioelectron, 25*(6), 1290-1294.

Djordjevic, M. (2007). SELEX experiments new prospects, applications and data analysis in inferring regulatory pathways. *Biomolecular Engineering, 24*(2), 179-189.

Du, Y., Chen, C., Li, B., Zhou, M., Wang, E., & Dong, S. (2010). Layer-by-layer electrochemical biosensor with aptamer-appended active polyelectrolyte multilayer for sensitive protein determination. *Biosens Bioelectron, 25*(8), 1902-1907.

Evtugyn, G. A., Porfireva, A. V., Hianik, T., Cheburova, M. S., & Budnikov, H. C. (2008). Potentiometric DNA Sensor Based on Electropolymerized Phenothiazines for Protein Detection. *Electroanalysis, 20*(12), 1300-1308.

Fabre, B., Samorì, C., & Bianco, A. (2012). Immobilization of double functionalized carbon nanotubes on glassy carbon electrodes for the electrochemical sensing of the biotin–avidin affinity. *Journal of Electroanalytical Chemistry, 665*, 90-94.

Fan, H., Li, H., Wang, Q., He, P., & Fang, Y. (2012). A host-guest-recognition-based electrochemical aptasensor for thrombin detection. *Biosens Bioelectron, 35*(1), 33-36.

Feng, K., Sun, C., Kang, Y., Chen, J., Jiang, J.-H., Shen, G.-L., & Yu, R.-Q. (2008). Label-free electrochemical detection of nanomolar adenosine based on target-induced aptamer displacement. *Electrochemistry Communications, 10*(4), 531-535.

Fisher, L. W., Torchia, D. A., Fohr, B., Young, M. F., & Fedarko, N. S. (2001). Flexible structures of SIBLING proteins, bone sialoprotein, and osteopontin. *Biochem Biophys Res Commun, 280*(2), 460-465.

Frense, D., Kang, S., Schieke, K., Reich, P., Barthel, A., Pliquett, U., Beckmann, D. (2013). Label-free impedimetric biosensor for thrombin using the thrombin-binding aptamer as receptor. *Journal of Physics: Conference Series, 434*, 012091.

General, I. J., Dragomirova, R., & Meirovitch, H. (2011). New method for calculating the absolute free energy of binding: the effect of a mobile loop on the avidin/biotin complex. *J Phys Chem B, 115*(1), 168-175.

Gokulrangan, G., Unruh, J. R., Holub, D. F., Ingram, B., Johnson, C. K., & Wilson, G. S. (2005). DNA Aptamer-Based Bioanalysis of IgE by Fluorescence Anisotropy. *Analytical Chemistry, 77*(7), 1963-1970.

Gopinath, S. C., Awazu, K., & Fujimaki, M. (2012). Waveguide-mode sensors as aptasensors. *Sensors (Basel), 12*(2), 2136-2151.

Grieshaber, D., MacKenzie, R., Voros, J., & Reimhult, E. (2008). Electrochemical Biosensors - Sensor Principles and Architectures. *Sensors, 8*, 1400-1458.

Guo, K.-T., Paul, A., Schichor, C., Ziemer, G., & Wendel, H. P. (2008). CELL-SELEX Novel Perspectives of Aptamer-Based Therapeutics. *International Journal of Molecular Sciences, 9*, 668-678.

Guo, K., Wang, Y., Chen, H., Ji, J., Zhang, S., Kong, J., & Liu, B. (2011). An aptamer–SWNT biosensor for sensitive detection of protein via mediated signal transduction. *Electrochemistry Communications, 13*(7), 707-710.

Guttridge, D. C. (1997). Thrombin Causes a Marked Delay in Skeletal Myogenesis That Correlates with the Delayed Expression of Myogenin and p21CIP1/WAF1. *Journal of Biological Chemistry, 272*(39), 24117-24120.

Hamaguchi, N., Ellington, A., & Stanton, M. (2001). Aptamer beacons for the direct detection of proteins. *Anal Biochem, 294*(2), 126-131.

Hamula, C., Guthrie, J., Zhang, H., Li, X., & Le, X. (2006). Selection and analytical applications of aptamers. *TrAC Trends in Analytical Chemistry, 25*(7), 681-691.

Han, K., Liang, Z., & Zhou, N. (2010). Design strategies for aptamer-based biosensors. *Sensors (Basel), 10*(5), 4541-4557.

Hansen, J. A., Wang, J., Kawde, A.-N., Xiang, Y., Gothelf, K. V., & Collins, G. (2006). Quantum-Dot/Aptamer-Based Ultrasensitive Multi-Analyte Electrochemical Biosensor *JACS articles, 128*, 2228-2229.

He, P., Shen, L., Cao, Y., & Li, D. (2007). Ultrasensitive Electrochemical Detection of Proteins by Amplification of Aptamer-Nanoparticle Bio Bar Codes. *Anal. Chem., 79*, 8024-8029.

Hianik, T., Ostatna, V., Sonlajtnerova, M., & Grman, I. (2007). Influence of ionic strength, pH and aptamer configuration for binding affinity to thrombin. *Bioelectrochemistry, 70*(1), 127-133.

Hianik, T., Ostatna, V., Zajacova, Z., Stoikova, E., & Evtugyn, G. (2005). Detection of aptamer-protein interactions using QCM and electrochemical indicator methods. *Bioorg Med Chem Lett, 15*(2), 291-295.

Higashikawa, F., Eboshida, A., & Yokosaki, Y. (2007). Enhanced biological activity of polymeric osteopontin. *FEBS Letters, 581*, 2697-2701.

Ho, H.-A., & Leclerc, M. (2004). Optical Sensors Based on Hybrid Aptamer/Conjugated Polymer Complexes. *JACS articles, 126*, 1384-1387.

Hong, P., Li, W., & Li, J. (2012). Applications of aptasensors in clinical diagnostics. *Sensors (Basel), 12*(2), 1181-1193.

Huang, D. W., Niu, C. G., Qin, P. Z., Ruan, M., & Zeng, G. M. (2010). Time-resolved fluorescence aptamer-based sandwich assay for thrombin detection. *Talanta, 83*(1), 185-189.

Huang, Y., Zhao, S., Chen, Z. F., Shi, M., Chen, J., & Liang, H. (2012). An amplified chemiluminescence aptasensor based on bi-resonance energy transfer on gold nanoparticles and exonuclease III-catalyzed target recycling. *Chem Commun (Camb), 48*(97), 11877-11879.

Ikebukuro, K., Kiyohara, C., & Sode, K. (2005). Novel electrochemical sensor system for protein using the aptamers in sandwich manner. *Biosens Bioelectron, 20*(10), 2168-2172.

Jayasena, S. D. (1999). Aptamers An Emerging Class of Molecules That Rival Antibodies in Diagnostics, *Clinical Chemistry, 45*(9), 1628-1650.

Ji, J., Gan, J., Kong, J., Yang, P., Liu, B., & Ji, C. (2012). Electrochemical detection of the activities of thrombin and its inhibitor. *Electrochemistry Communications, 16*(1), 53-56.

Jin, Y., Bai, J., & Li, H. (2010). Label-free protein recognition using aptamer-based fluorescence assay. *Analyst, 135*(7), 1731-1735.

Justino, C. I. L., Rocha-Santos, T. A., & Duarte, A. C. (2010). Review of analytical figures of merit of sensors and biosensors in clinical applications. *TrAC Trends in Analytical Chemistry, 29*(10), 1172-1183.

Kadkol, S. S., Lin, A. Y., Barak, V., Kalickman, I., Leach, L., Valyi-Nagy, K., Pe'er, J. (2006). Osteopontin expression and serum levels in metastatic uveal melanoma: a pilot study. *Investigative ophthalmology & visual science, 47*, 802-806.

Kanwar, J. R., Roy, K., & Kanwar, R. K. (2011). Chimeric aptamers in cancer cell-targeted drug delivery. *Crit Rev Biochem Mol Biol, 46*(6), 459-477.

Ke, H. L., Chang, L. L., Yang, S. F., Lin, H. H., Li, C. C., Wu, D. C., & Wu, W. J. (2011). Osteopontin overexpression predicts poor prognosis of upper urinary tract urothelial carcinoma. *Urol Oncol, 29*(6), 703-709.

Keefe, A. D., & Cload, S. T. (2008). SELEX with modified nucleotides. *Curr Opin Chem Biol, 12*(4), 448-456.

Khezrian, S., Salimi, A., Teymourian, H., & Hallaj, R. (2013). Label-free electrochemical IgE aptasensor based on covalent attachment of aptamer onto multiwalled carbon nanotubes/ionic liquid/chitosan nanocomposite modified electrode. *Biosens Bioelectron, 43*, 218-225.

Kim, J.-h., Skates, S. J., Wong, K.-k., Feltmate, C. M., Berkowitz, R. S., Cramer, D. W., & Mok, S. C. (2002). Osteopontin as a Potential Diagnostic Biomarker for Ovarian Cancer. *287*, 1671-1679.

Kim, Y. S., Lee, S. J., & Gu, M. B. (2008). Electrochemical Aptamer-based Biosensors. *2*, 175-182.

Kulbachinskiy, A. V. (2007). Methods for selection of aptamers to protein targets. *Biochemistry (Moscow), 72*(13), 1505-1518.

Kuralay, F., Campuzano, S., & Wang, J. (2012). Greatly extended storage stability of electrochemical DNA biosensors using ternary thiolated self-assembled monolayers. *Talanta, 99*, 155-160.

Kwon, D., Jeong, H., & Chung, B. H. (2011). Label-free electrochemical detection of human alpha-thrombin in blood serum using ferrocene-coated gold nanoparticles. *Biosens Bioelectron, 28*(1), 454-458.

Labuda, J., Brett, A. M. O., Evtugyn, G., Fojta, M., Mascini, M., Ozsoz, M., Wang, J. (2010). Electrochemical nucleic acid-based biosensors: Concepts, terms, and methodology (IUPAC Technical Report). *Pure and Applied Chemistry, 82*(5), 1161-1187.

Lee, J. A., Hwang, S., Lee, K.-C., Kwak, J., Park, S. I., & Lee, S. S. (2007). Pyrolyzed Carbon Biosensor for Aptamer-Protein Interactions Using Electrochemical Impedance Spectroscopy.

Lee, J. H., Wernette, D. P., Yigit, M. V., Liu, J., Wang, Z., & Lu, Y. (2007). Site-Specific Control of Distances between Gold Nanoparticles Using Phosphorothioate Anchors on DNA and a Short Bifunctional Molecular Fastener. *Angewandte Chemie, 119*(47), 9164-9168.

Lee, J. H., Yigit, M. V., Mazumdar, D., & Lu, Y. (2010). Molecular diagnostic and drug delivery agents based on aptamer-nanomaterial conjugates.*Adv Drug Deliv Rev, 62*(6), 592-605.

Lee, J. O., So, H. M., Jeon, E. K., Chang, H., Won, K., & Kim, Y. H. (2008). Aptamers as molecular recognition elements for electrical nanobiosensors. *Anal Bioanal Chem, 390*(4), 1023-1032.

Lee, M., & Walt, D. R. (2000). A fiber-optic microarray biosensor using aptamers as receptors. *Anal Biochem, 282*(1), 142-146.

Li, J., Orlandi, R., White, C. N., Rosenzweig, J., Zhao, J., Seregni, E., Chan, D. W. (2005). Independent validation of candidate breast cancer serum biomarkers identified by mass spectrometry. *Clin Chem, 51*(12), 2229-2235.

Li, J. J., Fang, X., & Tan, W. (2002). Molecular Aptamer Beacons for Real-Time Protein Recognition. *Biochem Biophys Res Commun, 292*(1), 31-40.

Li, W., Wang, K., Tan, W., Ma, C., & Yang, X. (2007). Aptamer-based analysis of angiogenin by fluorescence anisotropy. *Analyst, 132*(2), 107-113.

Lin, S. F., Ding, T. J., Liu, J. T., Lee, C. C., Yang, T. H., Chen, W. Y., & Chang, J. Y. (2011). A guided mode resonance aptasensor for thrombin detection. *Sensors (Basel), 11*(9), 8953-8965.

Liu, S., Zhang, X., Luo, W., Wang, Z., Guo, X., Steigerwald, M. L., & Fang, X. (2011). Single-Molecule Detection of Proteins Using Aptamer-Functionalized Molecular Electronic Devices. *Angewandte Chemie*.

Liu, Y., Tuleouva, N., Ramanculov, E., & Revzin, A. (2010). Aptamer-based electrochemical biosensor for interferon gamma detection. *Anal Chem, 82*(19), 8131-8136.

Liu, Z., Yuan, R., Chai, Y., Zhuo, Y., Hong, C., Yang, X., Qian, X. (2009). Highly sensitive, reusable electrochemical aptasensor for adenosine. *Electrochimica Acta, 54*(26), 6207-6211.

Lord, S. J., Lei, W., Craft, P., Cawson, J. N., Morris, I., Walleser, S., Houssami, N. (2007). A systematic review of the effectiveness of magnetic resonance imaging (MRI) as an addition to mammography and ultrasound in screening young women at high risk of breast cancer. *Eur J Cancer, 43*(13), 1905-1917.

Lu, Y., Zhu, N., Yu, P., & Mao, L. (2008). Aptamer-based electrochemical sensors that are not based on the target binding-induced conformational change of aptamers. *Analyst, 133*(9), 1256-1260.

Macrì, A., Versaci, A., Lupo, G., Trimarchi, G., Tomasello, C., Loddo, S., Famulari, C. (2009). Role of osteopontin in breast cancer patients. *95*, 48-52.

Mairal, T., Ozalp, V. C., Lozano Sanchez, P., Mir, M., Katakis, I., & O'Sullivan, C. K. (2008). Aptamers: molecular tools for analytical applications. *Anal Bioanal Chem, 390*(4), 989-1007.

Mathelin, C., Cromer, A., Wendling, C., Tomasetto, C., & Rio, M. C. (2006). Serum biomarkers for detection of breast cancers: A prospective study. *Breast Cancer Res Treat, 96*(1), 83-90.

Matsuura, M., Suzuki, T., & Saito, T. (2010). Osteopontin is a new target molecule for ovarian clear cell carcinoma therapy. *Cancer Sci, 101*(8), 1828-1833.

Mayer, G. (2009). The Chemical Biology of Aptamers. *Angewandte Chemie International Edition, 48*(15), 2672-2689.

McShane, L. M., Altman, D. G., Sauerbrei, W., Taube, S. E., Gion, M., & Clark, G. M. (2005). Reporting recommendations for tumor marker prognostic studies. *J Clin Oncol, 23*(36), 9067-9072.

Medley, C. D., Bamrungsap, S., Tan, W., & Smith, J. E. (2011). Aptamer-conjugated nanoparticles for cancer cell detection. *Anal Chem, 83*(3), 727-734.

Mi, Z., Guo, H., & Kuo, P. C. (2009). Identification of osteopontin-dependent signaling pathways in a mouse model of human breast cancer. *BMC Res Notes, 2*, 119.

Mi, Z., Guo, H., Russell, M. B., Liu, Y., Sullenger, B. a., & Kuo, P. C. (2009). RNA aptamer blockade of osteopontin inhibits growth and metastasis of MDA-MB231 breast cancer cells. *Molecular therapy : the journal of the American Society of Gene Therapy, 17*, 153-161.

Minunni, M., Tombelli, S., Gullotto, A., Luzi, E., & Mascini, M. (2004). Development of biosensors with aptamers as bio-recognition element: the case of HIV-1 Tat protein. *Biosens Bioelectron, 20*(6), 1149-1156.

Mir, M., Vreeke, M., & Katakis, I. (2006). Different strategies to develop an electrochemical thrombin aptasensor. *Electrochemistry Communications, 8*(3), 505-511.

Mirza, M., Shaughnessy, E., Hurley, J. K., Vanpatten, K. A., Pestano, G. A., He, B., & Weber, G. F. (2008). Osteopontin-c is a selective marker of breast cancer. *Int J Cancer, 122*(4), 889-897.

Misek, D. E., & Kim, E. H. (2011). Protein biomarkers for the early detection of breast cancer. *Int J Proteomics, 2011*, 343582.

Neundlinger, I., Poturnayova, A., Karpisova, I., Rankl, C., Hinterdorfer, P., Snejdarkova, M., Ebner, A. (2011). Characterization of enhanced monovalent and bivalent thrombin DNA aptamer binding using single molecule force spectroscopy. *Biophys J, 101*(7), 1781-1787.

Ng, J. K.-K., & Chong, S. S. (2011). Multiplexing Capabilities of Biosensors for Clinical Diagnostics *Biosensors for Health, Environment and Biosecurity*.

Numnuam, A., Chumbimuni-Torres, K. Y., Xiang, Y., Bash, R., Thavarungkul, P., Kanatharana, P., Bakker, E. (2008). Potentiometric Detection of DNA Hybridization. *J Am Chem Soc., 130*(2), 410–411.

Nutiu, R., & Li, Y. (2004). Structure-switching signaling aptamers: transducing molecular recognition into fluorescence signaling. *Chemistry, 10*(8), 1868-1876.

O'Sullivan, C. K. (2002). Aptasensors–the future of biosensing? *Anal Bioanal Chem, 372*(1), 44-48.

Ocana, C., Pacios, M., & del Valle, M. (2012). A reusable impedimetric aptasensor for detection of thrombin employing a graphite-epoxy composite electrode. *Sensors (Basel), 12*(3), 3037-3048.

Opstal-van Winden, A. W., Krop, E. J., Karedal, M. H., Gast, M. C., Lindh, C. H., Jeppsson, M. C., Vermeulen, R. C. (2011). Searching for early breast cancer biomarkers by serum protein profiling of pre-diagnostic serum; a nested case-control study. *BMC Cancer, 11*, 381.

Orita, T., Tomita, M., Harada, M., & Kato, K. (2012). Binding activity of avidin to the biotin within mesoporous silica materials for bioanalytical applications. *Anal Biochem, 425*(1), 1-9.

Paborsky, L. R., N. McCurd, S., C. Griffin, L., J. Toole, J., & L. K. Leung, L. (1993). The Single-stranded DNA Aptamer-binding Site of Human Thrombin. *The Journalmof biological chemistry, 288*(28), 20808-20811.

Park, M. G., Oh, M. M., Yoon, J. H., Park, J. Y., Park, H. S., Moon, D. G., & Yoon, D. K. (2012). The value of plasma osteopontin levels as a predictive factor of disease stage and recurrence in patients with bladder urothelial carcinoma: A prospective study. *The Kaohsiung Journal of Medical Sciences*.

Phillips, J. A., Xu, Y., Xia, Z., Fan, Z. H., & Tan, W. (2009). Enrichment of cancer cells using aptamers immobilized on a microfluidic channel. *Anal Chem, 81*(3), 1033-1039.

Potyrailo RA, Conrad RC, Ellington AD, & GM., H. (1998). Adapting selected nucleic acid ligands (aptamers) to biosensors. *Anal Chem., 70*(16), 3419-3425.

Radi, A.-E. (2011). Electrochemical Aptamer-Based Biosensors: Recent Advances and Perspectives. *International Journal of Electrochemistry, 2011*, 1-17.

Radi, A.-E., Sánchez, J. L. A., Baldrich, E., & O`Sullivan, C. K. (2005). Reagentless, Reusable, Ultrasensitive Electrochemical Molecular Beacon Aptasensoral *JACS articles, 128*, 117-124.

Radi, A. E., Acero Sanchez, J. L., Baldrich, E., & O'Sullivan, C. K. (2005). Reusable impedimetric aptasensor. *Anal Chem, 77*(19), 6320-6323.

Radi, A. E., Acero Sanchez, J. L., Baldrich, E., & O'Sullivan, C. K. (2006). Reagentless, reusable, ultrasensitive electrochemical molecular beacon aptasensor. *J Am Chem Soc, 128*(1), 117-124.

Rangaswami, H., Bulbule, A., & Kundu, G. C. (2006). Osteopontin: role in cell signaling and cancer progression. *Trends Cell Biol, 16*(2), 79-87.

Remmele, R. L. J., Enk, J. Z.-v., Dharmavaram, V., Balaban, D., Durst, M., Shoshitaishvili, A., & Rand, H. (2005). Scan-Rate-Dependent Melting Transitions of Interleukin-1 Receptor (Type II): Elucidation of Meaningful Thermodynamic and Kinetic Parameters of Aggregation Acquired from DSC Simulations. *JACS articles, 127* 8328-8339.

Rittling, S. R., & Chambers, A. F. (2004). Role of osteopontin in tumour progression. *Br J Cancer, 90*(10), 1877-1881.

Rodrigues, L. R., Lopes, N., Sousa, B., Vieira, D., & Milanezi, F. (2009). Significance of Osteopontin Expression in Human Invasive Breast Tumour Stroma. 1-9.

Rodrigues, L. R., Teixeira, J. a., Schmitt, F. L., Paulsson, M., & Lindmark-Mänsson, H. (2007). The role of osteopontin in tumor progression and metastasis in breast cancer. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology, 16*, 1087-1097.

Ronkainen, N. J., Halsall, H. B., & Heineman, W. R. (2010). Electrochemical biosensors. *Chem Soc Rev, 39*(5), 1747-1763.

Rudland, P. S., Platt-higgins, A., El-tanani, M., Rudland, S. D. S., Barraclough, R., Winstanley, J. H. R., West, C. R. (2002). Prognostic Significance of the Metastasis-associated Protein Osteopontin in Human Breast Cancer 1. 3417-3427.

Rusmini, F., Zhong, Z., & Feijen, J. (2007). Protein immobilization strategies for protein biochips. *Biomacromolecules, 8*, 1775-1789.

Ryder, S. P., Recht, M. I., & Williamson, J. R. (2008). Quantitative analysis of protein-RNA interactions by gel mobility shift. *Methods Mol Biol, 488*, 99-115.

Sadana, A., & Sadana, N. (2011). Handbook of Biosensors and Biosensor Kinetics.

Sassolas, A., Blum, L. J., & Leca-Bouvier, B. D. (2009). Electrochemical Aptasensors. *Electroanalysis, 21*(11), 1237-1250.

Sassolas, A., Blum, L. J., & Leca-Bouvier, B. D. (2011). Optical detection systems using immobilized aptamers. *Biosens Bioelectron*.

Sassolas, A., Leca-Bouvier, B. D., & Blum, L. c. J. (2008). DNA Biosensors and Microarrays. *Chemical Review, 108*, 108-139.

Servais, E. L., Suzuki, K., Colovos, C., Rodriguez, L., Sima, C., Fleisher, M., Adusumilli, P. S. (2011). An in vivo platform for tumor biomarker assessment. *PLoS One, 6*(10), e26722.

Shojaei, F., Scott, N., Kang, X., Lappin, P. B., Fitzgerald, A. A., Karlicek, S., Kraynov, E. (2012). Osteopontin induces growth of metastatic tumors in a preclinical model of non-small lung cancer. *J Exp Clin Cancer Res, 31*, 26.

Song, K. M., Lee, S., & Ban, C. (2012). Aptamers and their biological applications. *Sensors (Basel), 12*(1), 612-631.

Song, S., Wang, L., Li, J., Fan, C., & Zhao, J. (2008). Aptamer-based biosensors. *TrAC Trends in Analytical Chemistry, 27*(2), 108-117.

Song, S., Xu, H., & Fan, C. (2006). Potential diagnostic applications of biosensors: current and future directions. *International Journal of Nanomedicine 1*(4), 433–440.

Sosic, A., Meneghello, A., Cretaio, E., & Gatto, B. (2011). Human thrombin detection through a sandwich aptamer microarray: interaction analysis in solution and in solid phase. *Sensors (Basel), 11*(10), 9426-9441.

Stojanovic, M. N., Prada, P. D., & Landry, D. W. (2000). Fluorescent sensors based on aptamer self-assembly. *J. Am. Chem. Soc., 122*, 11547–11548.

Stoltenburg, R., Reinemann, C., & Strehlitz, B. (2007). SELEX–a (r)evolutionary method to generate high-affinity nucleic acid ligands. *Biomol Eng, 24*(4), 381-403.

Strehlitz, B., Nikolaus, N., & Stoltenburg, R. (2008). Protein Detection with Aptamer Biosensors. *Sensors, 8*(7), 4296-4307.

Strehlitz, B., Reinemann, C., Linkorn, S., & Stoltenburg, R. (2011). Aptamers for pharmaceuticals and their application in environmental analytics. *Bioanal Rev, 4*(1), 1-30.

Sultan, Y., Walsh, R., Monreal, C., & DeRosa, M. C. (2009). Preparation of functional aptamer films using layer-by-layer self-assembly. *Biomacromolecules, 10*(5), 1149-1154.

Suprun, E., Shumyantseva, V., Bulko, T., Rachmetova, S., Rad'ko, S., Bodoev, N., & Archakov, A. (2008). Au-nanoparticles as an electrochemical sensing platform for aptamer-thrombin interaction. *Biosens Bioelectron, 24*(4), 831-836.

Tang, C.-F., & Shafer, R. H. (2006). Engineering the Quadruplex Fold: Nucleoside Conformation Determines Both Folding Topology and Molecularity in Guanine Quadruplexes. *J. Am. Chem. Soc., 128*(17), 5966–5973.

Tasset, D. M., Kubik, M. F., & Steiner, W. (1997). Oligonucleotide Inhibitors of Human Thrombin that Bind Distinct Epitopes. *J. Mol. Biol. , 272*, 688±698.

Thevenot, D. R., Toth, K., Durst, R. A., & Wilson, G. S. (1999). Electrochemical biosensors Recommended definitions and classification (Technical Report). *Pure Appl. Chem., 71*(12), 2333±2348.

Thévenot, D. R., Toth, K., Durst, R. A., & Wilson, G. S. (2001). Electrochemical biosensors: recommended definitions and classification. *Biosensors & Bioelectronics 16*, 121-131.

Tombelli, S., Minunni, M., Luzi, E., & Mascini, M. (2005). Aptamer-based biosensors for the detection of HIV-1 Tat protein. *Bioelectrochemistry, 67*(2), 135-141.

Tothill, I. E. (2009). Biosensors for cancer markers diagnosis.*Semin Cell Dev Biol, 20*(1), 55-62.

Tran, D. T., Janssen, K. P., Pollet, J., Lammertyn, E., Anne, J., Van Schepdael, A., & Lammertyn, J. (2010). Selection and characterization of DNA aptamers for egg white lysozyme. *Molecules, 15*(3), 1127-1140.

Ulrich, H., & Wrenger, C. (2009). Disease-specific biomarker discovery by aptamers. *Cytometry A, 75*(9), 727-733.

Velasco-Garcia, M., & Missailidis, S. (2009). New trends in aptamer-based electrochemical biosensors. *Gene Therapy and Molecular Biology, 13*(1), 1-10.

Wai, P. Y., & Kuo, P. C. (2004). The Role of Osteopontin in Tumor Metastasis. *Journal of Surgical Research, 121*, 228-241.

Wai, P. Y., & Kuo, P. C. (2008). Osteopontin: regulation in tumor metastasis. *Cancer Metastasis Rev, 27*(1), 103-118.

Wang, J. (2006). Electrochemical biosensors: towards point-of-care cancer diagnostics. *Biosens Bioelectron, 21*(10), 1887-1892.

Wang, J., Chen, G., Jiang, H., Li, Z., & Wang, X. (2013). Advances in nano-scaled biosensors for biomedical applications. *Analyst*.

Wang, P., Yang, Y., Hong, H., Zhang, Y., Cai, W., & Fang, D. (2011). Aptamers as Therapeutics in Cardiovascular Diseases. *Curr Med Chem., 18*(27), 4169-4174.

Wang, W., Chen, C., Qian, M., & Zhao, X. S. (2008). Aptamer biosensor for protein detection using gold nanoparticles. *Anal Biochem, 373*(2), 213-219.

Wang, X. Y., Gao, A., Lu, C. C., He, X. W., & Yin, X. B. (2013). An electrochemiluminescence aptasensor for thrombin using graphene oxide to immobilize the aptamer and the intercalated probe. *Biosens Bioelectron, 48*, 120-125.

Weber, G. F., Lett, G. S., & Haubein, N. C. (2010). Osteopontin is a marker for cancer aggressiveness and patient survival. *Br J Cancer, 103*(6), 861-869.

Wei, D., Bailey, M. J., Andrew, P., & Ryhanen, T. (2009). Electrochemical biosensors at the nanoscale. *Lab Chip, 9*(15), 2123-2131.

Wei, F., Lillehoj, P. B., & Ho, C. M. (2010). DNA diagnostics: nanotechnology-enhanced electrochemical detection of nucleic acids. *Pediatr Res, 67*(5), 458-468.

Wu, Z. S., Zheng, F., Shen, G. L., & Yu, R. Q. (2009). A hairpin aptamer-based electrochemical biosensing platform for the sensitive detection of proteins. *Biomaterials, 30*(15), 2950-2955.

Xiao, Y., Lubin, A. A., Heeger, A. J., & Plaxco, K. W. (2005). Label-free electronic detection of thrombin in blood serum by using an aptamer-based sensor. *Angew Chem Int Ed Engl, 44*(34), 5456-5459.

Xie, S., & Walton, S. P. (2010). Development of a dual-aptamer-based multiplex protein biosensor. *Biosens Bioelectron, 25*(12), 2663-2668.

Xie, S., Yuan, R., Chai, Y., Bai, L., Yuan, Y., & Wang, Y. (2012). Label-free electrochemical aptasensor for sensitive thrombin detection using layer-by-layer self-assembled multilayers with toluidine blue-graphene composites and gold nanoparticles. *Talanta, 98*, 7-13.

Xie, W. Z., Leibl, M., Clark, M. R., Dohrmann, P., Kunze, T., & Gieseler, F. (2005). Activation of the coagulation system in cancerogenesis and metastasation. *Biomed Pharmacother, 59*(3), 70-75.

Xu, D., Xu, D., Yu, X., Liu, Z., He, W., & Ma, Z. (2005). Label-free Electrochemical Detection for aptamer-Based Array Electrodes. *Anal Chem, 77*, 5107-5113.

Xu, Y., Cheng, G., He, P., & Fang, Y. (2009). A Review: Electrochemical Aptasensors with Various Detection Strategies. *Electroanalysis, 21*(11), 1251-1259.

Xu, Y., Yang, L., Ye, X., He, P., & Fang, Y. (2006). An Aptamer-Based Protein Biosensor by Detecting the Amplified Impedance Signal. *Electroanalysis, 18*(15), 1449-1456.

Yan, F., Wang, F., & Chen, Z. (2011). Aptamer-based electrochemical biosensor for label-free voltammetric detection of thrombin and adenosine. *Sensors and Actuators B: Chemical, 160*(1), 1380-1385.

Zhang, Y.-L., Huang, Y., Jiang, J.-H., Shen, G.-L., & Yu, R.-Q. (2007). Electrochemical Aptasensor Based on Proximity-Dependent Surface Hybridization Assay for Single-Step, Reusable, Sensitive Protein Detection. *JACS articles, 129*, 15448-15449.

Zhao, J., Zhang, Y., Li, H., Wen, Y., Fan, X., Lin, F., Yao, S. (2011). Ultrasensitive electrochemical aptasensor for thrombin based on the amplification of aptamer-AuNPs-HRP conjugates. *Biosens Bioelectron, 26*(5), 2297-2303.

Zhao(a), J., He, X., Bo, B., Liu, X., Yin, Y., & Li, G. (2012). A "signal-on" electrochemical aptasensor for simultaneous detection of two tumor markers. *Biosens Bioelectron, 34*(1), 249-252.

Zhou, L., Wang, M.-H., Wang, J.-P., & Ye, Z.-Z. (2011). Application of Biosensor Surface Immobilization Methods for Aptamer. *Chinese Journal of Analytical Chemistry, 39*, 432-438.

Zhou, Y., Chiu, C. W., & Liang, H. (2012). Interfacial structures and properties of organic materials for biosensors: an overview. *Sensors (Basel), 12*(11), 15036-15062.

Zou, M., Chen, Y., Xu, X., Huang, H., Liu, F., & Li, N. (2012). The homogeneous fluorescence anisotropic sensing of salivary lysozyme using the 6-carboxyfluorescein-labeled DNA aptamer. *Biosens Bioelectron, 32*(1), 148-154.

Zuo, X., Xiao, Y., & Plaxco, K. W. (2009). High specificity, electrochemical sandwich assays based on single aptamer sequences and suitable for the direct detection of small-molecule targets in blood and other complex matrices. *J. Am. Chem. Soc., 131*, 6944–6945.